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SANTA BARBARA • SANTA CRUZ

COLLEGE OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES
CALIFORNIA INSTITUTE OF FOOD AND AGRICULTURAL RESEARCH
258 CRUESS HALL
DAVIS, CA 95616-8598
(916) 752-2922
FAX (916) 752-4759

November 30, 1993

National Renewable Energy Laboratory
Attn: Michael Himmel
1617 Cole Blvd.
Golden, CO 80401-3393

Dear Mike,

Enclosed is the final Technical Status Report for subcontract XA-1-11225-1 for the period, November 15 - November 30, 1993. Please note that this report represents a 15 day period.

Sincerely,

Sharon P. Shoemaker

Sharon P. Shoemaker, Ph.D.

SPS/pg
Enclosure

Characterization of endo-1,4-beta-glucanase by viscometry and reducing end-group formation

NREL Subcontract XA-1-11225-1

Final Report

California Institute of Food and Agricultural Research

University of California, Davis
October 1993

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1. INTRODUCTION

The commercial viability of processes for the conversion of low-value cellulosic biomass to ethanol depends on the improvement of production and activity of cellulases. The fundamental and rate limiting step in processes based on the use of bacterial and fungal systems is depolymerization of crystalline cellulose.

The multi-enzyme cellulase system is composed mainly of:
- endoglucanases (endo-1.4-ß-D-glucan glucanohydrolases, EC 3.2.1.4);
- cellobiohydrolases (1,4-ß-D-glucan cellobiohydrolases, EC 3.2.1.91);
- ß-glucosidase (ß-D-glucoside glucohydrolase, EC 3.2.1.21).

The first group constitutes endo-splitting enzymes, whereas the second and third constitute exo-splitting enzymes.

The insolubility and structural variation of the natural cellulose presents a particular difficulty to the study of cellulases. Moreover, additional problems are associated with the complexity of the system. There exists within the cellulase research community considerable controversy regarding the actual activity data reported in the literature. In particular, the assessment of endoglucanase activity is a matter of concern, which leads to the need of adoption of assay methods that deserve a high degree of confidence.

Endoglucanases have been characterized using the soluble substrates, carboxymethylcellulose (CMC) or hydroxyethylcellulose (HEC), and the assays are based on:
- rate of formation of reducing end-groups
- rate of viscosity reduction

Methods based on reducing end-groups vary in sensitivity and no method distinguishes the position along the polymer chain where the linkage was broken and a reducing end-group was formed. Viscometric methods, on the other hand, provide such information and, therefore, are preferred for the assessment of endoglucanases, as for other depolymerases. There is a general acceptance of the viscometric method as being very sensitive and precise for the analysis of endoglucanases (Almin and Eriksson, 1967; Hulme, 1971; Almin et al. 1975; Guignard and Pilet, 1976; Coughlan and Folan, 1979; Mullings, 1985). Nevertheless, viscometric results are typically expressed in relative units and there is a plethora of arbitrary units of enzymatic activity used of empirical relationships involving constants approximations of recognized equations. Moreover, it is not possible to analyze comparatively most of published data because of differences in experimental conditions and enzymes.

Hence, there continues to be a need for an informative, accurate, sensitive, yet reliable and practical method to facilitate the screening of potential sources of endoglucanases as well as evaluate new endoglucanases obtained using recombinant techniques. In addition, it would be desirable to count on methods allowing rheological characterization of endoglucanases using relevant substrates, such as natural cellulose, and various forms of plant biomass.

2. OBJECTIVES OF THE SUBCONTRACT

1- Identify and experimentally evaluate existing and new methods of endoglucanase assay based on viscometric measurements.

2- Finalize and publish the "Recommended Viscosity Assay Method" developed.

3- Use the rheological methodology developed to compare the activity of native and recombinant endoglucanases in pure form and in mixtures of pure enzymes.

4- Participate with NREL researchers in the parallel rheological and reductometric analyses of selected, purified endoglucanases from fungal and bacterial sources.

5- Consult with NREL researchers in the compilation of a data-base regarding important endoglucanases (from literature study and rheological assays).

3. BRIEF CONSIDERATIONS ON THE USE OF VISCOMETRY FOR THE DETERMINATION OF MOLECULAR WEIGHT

The viscosity reduction observed by the action of endoglucanases is followed by changes in drain times during the incubation of these enzymes with chemically modified soluble cellulose derivatives (such as CMC, HEC). The changes in fluidity ($1/\eta_{sp}$) per unit of time are frequently used as a measurement of cellulase activity. However, these results are given in relative units.

The initial hydrolysis products of endoglucanases have high molecular weight and constitute substrates for further attack by these enzymes. The sole method of following precisely the course of the substrate degradation involves investigations of the changes in molecular weight undergone by the substrate.

Viscometry is a relative technique for estimation of molecular weight. Although the experimental part of a viscometric analysis is simple, converting the data to molecular weights and comparison with other samples is not always a straightforward task.

The basis for using viscosity as a molecular weight sensitive method is the relation between the intrinsic viscosity (limiting viscosity number) [η] and an average of the molecular weight. The Mark-Houwink equation forms the basis for using viscosity to determine molecular weight:

$$[\eta]=K'.M^{a}$$

K' and *a* are empirical parameters which are constants at a given temperature, for a specified solvent within each polysaccharide for a limited range of molecular weights. For a given polysaccharide-solvent system, *K'* and *a* can be estimated, from the determination of molecular weight and intrinsic viscosity, using a series of calibration substances which should be nearly monodisperse. However, polydispersity is an inherent property of almost any polysaccharide preparation. Other difficulties arise when extrapolation of those calibrations are done, because also the total ionic strength in solution would affect the calibration parameters using the above equation. Then, the unknown sample should be characterized under the same solvent conditions as those used to obtain the calibration parameters.

In many situations, comparisons of endoglucanase activity are required; when using enzymes which have different optimal conditions of temperature and pH, a calibration should be done for each particular combination of experimental conditions. These problems limit the value of converting [η] to molecular weight, unless a comparison with absolute methods can be performed.

Moreover, the viscosities of solutions of polyelectrolytes (as CMC), especially when the molecular weight is high, usually vary with shear rate at the concentrations normally used to measure intrinsic viscosity. Therefore, when measurements of viscosity are performed to estimate molecular weight, it is imperative to adopt the same conditions of shear that were used when the constants *K'* and *a* were evaluated

(Miller, 1966).

Manning (1981) pointed out that the error in determining the intrinsic viscosity of non-Newtonian fluids is not diminished by extrapolation to infinite dilution. Only extrapolation of the observed viscosity to zero shear rate leads to an accurate value for intrinsic viscosity.

The apparent viscosity of a non-Newtonian solution is dependent upon the shear rate, which depends upon the volume of liquid flowing through a capillary of radius *r* in the time *t*. A series of drain time measurements using different viscometers of known capillary radius and efflux volume is required to determine the specific viscosity at zero shear rate, necessary for further calculations. It was considered (Almin and Eriksson, 1967; Manning, 1981) that the Huggins equation did not give as good fit of the data as the Baker relationship

$$\eta_{sp0} + 1 = (1 + [\eta].C_s)^n \qquad \text{(Baker relationship)}$$

where, η_{sp0} is the specific viscosity at zero shear rate; [η] is the intrinsic viscosity, C_s of the concentration of substrate and *n* is a constant experimentally determined.

For each new lot of substrate (CMC) that is put into use the necessary viscometric parameters should be obtained, namely *n* (used in the Baker relationship) and *K'* and *a* (used in the Mark-Houwink equation).

Basically, the above theory was used in the strategy recommended by Manning (1981) for the viscometric assessment of endoglucanase activity in absolute units. Variations appeared in the work of other research groups (Almin and Eriksson, 1967; Werner, 1969) that developed methods of expressing cellulase activity in absolute units. Hulme (1971) criticized Werner (1969) pointing out that Werner had introduced errors of considerable magnitude because of the many approximations made. Also Hulme (1971) considered the work of Almin and Eriksson (1967) and Almin et al. (1967) unnecessarily complex, and even somewhat inaccurate because of the introduction of an empirical relationship between intrinsic viscosity and time.

The method proposed by Hulme (1971) is a simple approach to estimate the activity of cellulase in absolute terms. From a plot of reduced viscosity (η_{sp}/ c) *versus* concentration, the intrinsic viscosity [η] and Huggins constant are determined:

$$\eta_{sp} / c = [\eta] + k_H [\eta]^2 . c \qquad \text{(Huggins equation)}$$

Hulme (1971) considered that his method uses "relatively insignificant approximations" providing a direct theoretical approach for the assay of any depolymerase acting randomly if the event can be viscometrically measured.

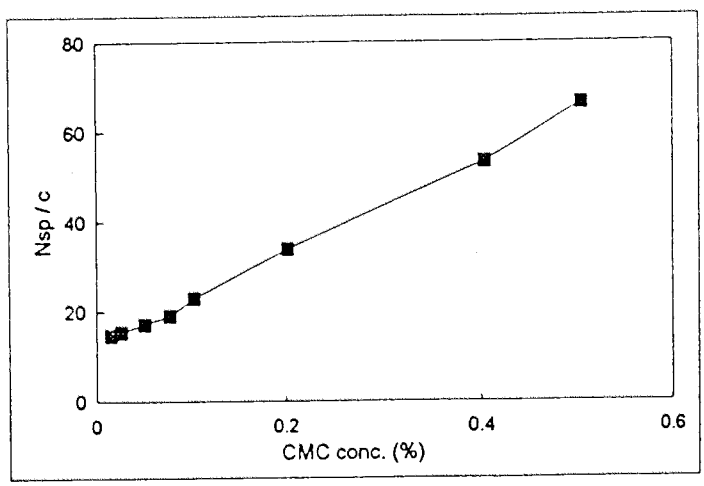
Indeed, it is a problem to adopt values for some of those constants reported in the literature. As a simple example, in the work of Hulme (1971) he used CMC (DS = 0.7) solutions prepared in 0.05 *M* acetate buffer at pH 5.0; the viscometric assays were run at 40°C. He reported a k_H value of 0.24 for the Huggins' constant. In our laboratory

Figure 1. Determination of the Huggins' constant from a series of CMC solutions prepared in Q05 M acetate buffer; viscosity measurements were carried out with Ubbelohde capillaries.

% CMC	Nsp	Nsp / c
0.01515	0.2215	14.62046
0.02525	0.387	15.32673
0.0505	0.8647	17.12277
0.07575	1.4419	19.03498
0.101	2.3027	22.79901
0.202	6.8259	33.79158
0.404	21.5979	53.46015
0.505	33.4661	66.2695

Regression Output:

Constant		12.17653
Std Err of Y Est		0.902168
R Squared		0.99817
No. of Observations		8
Degrees of Freedom		6
X Coefficient(s)	105.1847	
Std Err of Coef.	1.838418	



Huggins' equation:

$\eta_{sp} / c = [\eta] + K_H [\eta]^2 . c$

slope = $K_H [\eta]^2 = 105.1847$

$[\eta] = 12.17653$

$K_H = 105.1847 / 108.27$

$K_H = 0.709$

we estimated a k_H value of 0.709 (Figure 1). Basically the experimental conditions of the experiments were the same, except that Hulme used a CMC with a molecular weight of 70,000, while our CMC has an estimated molecular weight of 440,000. Werner (1969) reported a k_H value of 0.60, and he pointed out that the Huggins constant is independent of the molecular weight.

Rarely two investigators used the same type of polymer as a substrate for the endoglucanase analysis (Table 1), in addition, it should be considered that with poorly defined substrates, such as most soluble cellulose derivatives, there are variations between lots of polysaccharide. Also, it is not common to find a complete description of the substrate characteristics, which may lead to difficulties in comparing experimental conditions.

In a well conducted experiment the investigator might choose to measure the required constants instead of working with literature values. Nevertheless, it seems clear from the previous comments that this is not a simple task, it is a time-consuming one, and should be repeated whenever a new lot of substrate is used.

In order to partially simplify and particularly to validate the work, it is believed that the use of an absolute method of determining molecular weight is necessary, otherwise the value of converting [η] to molecular weight is quite limited.

Among the available methods, our choice is the use of a laser light scattering. The most direct method for measuring number average molecular weight (M_n) of a polymer solution is laser light scattering. Light scattering will yield M_n which is the key parameter in measuring the number of bonds hydrolyzed and its relationship to viscosity. With experimentally measured M_n's and viscosities of solutions of modified cellulose polymers, the validity of the various theories which attempt to relate viscosities to M_n's can be tested.

The use of a laser light scattering instrument as a detector on a gel permeation chromatographic column could provide an absolute measurement for characterization of endoglucanase activity. The gel permeation column separates by molecular weight the products of a hydrolyzed modified cellulose solution. With a light scattering detector, these products can be characterized by their absolute molecular weights. This can eliminate problems with calibration standards, ionic strength effects, and flow rate changes which occur when using conventional detectors such as refractive index detectors on chromatographic systems. With this methodology, the validity of a simplified method for measuring endoglucanase activity can be verified.

Table 1. Soluble cellulose derivatives used by different researchers in viscometric assessment of endoglucanase activity.

Source	Polymer	Type
Eriksson and Lindvall (1959)	CMC	DS[a] 0.89 DP[a] 150
Almin and Eriksson (1967)	CMC	DS DP 0.48 400 0.58 151 0.85 382 0.83 1890
Almin et a. (1967)	CMC	DS 0.83 DP 1890
Hulme (1971)	CMC	DS 0.7 MW 70,000
Child et al. (1973)	HEC	MS DP 1.0 563 2.0 750
Almin et al (1975)	CMC	DS 0.88
Sieben (1975)	CMC, HEC	
Demeester et al. (1979)	HEC	MS 2.5
Canevascini et al. (1979)	CMC	DS 0.57 - 0.87
Manning (1981)	CMC	DS 0.7 - 0.8
Esterbauer et al. (1985)	CMC	DS 0.7

[a] DS = degree of substitution; DP = degree of polymerization; MS = molar substitution

4. EXPERIMENTAL WORK

During the first year of the subcontract the experimental conditions for conducting the viscometric assays were reviewed, based on the literature and on our own experimental work. Basically, we had been using a capillary and a rotational (Brookfield) system, in parallel with a determination of reducing end-groups. An improved method based on the use of disodium 2.2' bicinchoninate (BCA assay) had been adapted for the evaluation of reducing end-groups. The resultant paper is being published by the *Journal of Food Biochemistry* (1993).

Concerning the use of viscometric analysis for the assessment of endoglucanases, our work has progressed but it is not concluded yet. Some of the viscometric parameters required to allow the evaluation of the changes in molecular weight of the substrate are being estimated. As it is probably clear from the comments presented in Section 3, this is not a simple task. Moreover, it is considered of fundamental importance the need of adopting an absolute method for evaluating molecular weight, in order to validate our results.

4.1 Assessment of endoglucanase activity

Our results on endoglucanase activity have been expressed based on changes in fluidity per unit of time and on production of reducing end-groups. Basically a capillary viscometer has been the instrument used for analytical purposes. Two other systems - rotational rheometers - have been compared to the capillary viscometer. These other instruments were tried in order to simplify the assays; they require smaller volumes of sample, also they allow the obtention of viscosity measurements at constant intervals, and immediately after loading the chamber with the solution.

Appendix 1 presents a description of the experimental protocols adopted with each particular instrument and method used.

4.1.1 Preparation of CMC solutions

The chemically modified soluble cellulose derivative selected for our experiments was carboxymethylcellulose CMC 7H3SF, obtained from Aqualon Co (formerly Hercules Inc., Willmington, DE). The CMC batch that has been used has a DS of 0.89, and an average molecular weight (M_n) of 440,000.

Considerable time and effort was spent initially in examining the soluble substrates CMC and HEC. Because of its non-ionic character, HEC would be an adequate substrate; however, HEC was eliminated as a choice because of foaming problems with the capillary viscometer.

Depending on the optimum pH of the endoglucanase to be assayed, the CMC solution is prepared in a pre-selected buffer. That being so, the following were the buffers used:

- 0.05 *M* sodium acetate buffer pH 5.0 for *T. reesei* EGI and EGII enzymes;
- 0.05 *M* sodium acetate buffer pH 5.5 for *T. fusca* E5 enzyme;
- 0.05 *M* sodium citrate buffer pH 5.8 for *A. cellulolyticus* E1 enzyme.

Substrate was dispersed in buffer with continuous stirring, followed by 1 h of agitation. The preparation was blended for 1 min and then heated for 1 h at 80°C with stirring. The preparation was filtered through medium sintered glass and degassed before use. In order to verify the exact final concentration of CMC, the dry weight of each preparation was determined. As required, dilutions of the substrates were prepared by weighing appropriate amounts of CMC solutions with the corresponding buffers.

4.1.2 Instrumentation

For the capillary viscosity measurements the system used is a Schott America automated viscometer adapted with Ubbelohde capillary tubes immersed in a temperature controlled water bath, and connected to an automated cleaner. This system is equipped with an autorecording optical detector for measurements of up to ten sequential readings. The Ubbelohde tubes were selected because their design minimizes surface tension effects. The capillary tube size is selected according to the concentration of the CMC solution to be used and to the temperature under which the measurements are taken. Following the recommendations of the manufacturer, the Ubbelohde capillary is loaded with 20 mL of buffer, or CMC, or CMC plus enzyme, by means of a syringe.

Two rotational systems have also been used:

- Brookfield viscometer DV III (Stoughton, MA), using an adapter (cat. SC4-18/13R) for samples of low viscosity; sample volume 8 mL;

- Carri-Med controlled stress rheometer (TA Instruments Co., Valleyview, Ohio), using a cone and plate fixture; sample volume 3-5 mL.

The rotational type viscometers are considered the most satisfactory for use with non-Newtonian fluids due to their accurate specified shearing conditions. However, at low concentration, CMC reveals a Newtonian behavior, especially at low shear rate, which allows the use of a capillary system.

The limitations of both rotational systems used refer to their sensitivity at low concentrations of CMC solutions, which should not be lower than 0.2% for the Brookfield, and not lower than 0.05% for the Carri-Med. However, in some instances these systems might be very advantageous because of the simplicity of their use and the quick obtention of measurements. Also, it should be pointed our that the Carri-Med is a very versatile system that allows the use of measuring devices with different geometries, such as parallel plates, which enables the study of suspensions containing large particles.

The results obtained with these rotational systems have been presented in viscosity reduction *vs.* time. Calculations of intrinsic viscosity and fluidity have not been performed because of the equipment limitations for the measurement of the viscosity of buffers.

4.1.3 BCA assay

As stated previously, the determination of reducing end-groups using BCA assay has significantly improved the sensitivity for detection of the hydrolytic activity of endoglucanases. Although reducing sugar assays are often considered as an adjunct to measurements of viscosity reduction in the assessment of endoglucanase activity, they should be considered essential, in order to relate rate of decrease in viscosity to the rate of glucosidic bonds hydrolyzed. Reductometry and viscometry cannot be regarded as alternative methods for the determination of endoglucanase activity. The measurement of the liberation of reducing sugars is not considered a typical measure of the random action of endoglucanases, while the drastic viscosity reduction observed in the initial attack of cellulosic substrate is.

Moreover, the BCA assay could be applied to the determination of the activity of cellulases on different cellulosic substrates. BCA assay use could even be extended to the evaluation of the activity of many other carbohydrases.

In addition, our lab has used the BCA assay to estimate the number-average molecular weight (M_n) of CMC. As CMC is polydisperse, it consists of a mixture of molecules having different chain lengths. From CMC solutions, where the dry weight is known, the number of reducing end-groups is determined by the BCA assay, and used used to calculate the M_n:

$$M_n = \text{dry weight/ moles reducing end-groups}$$

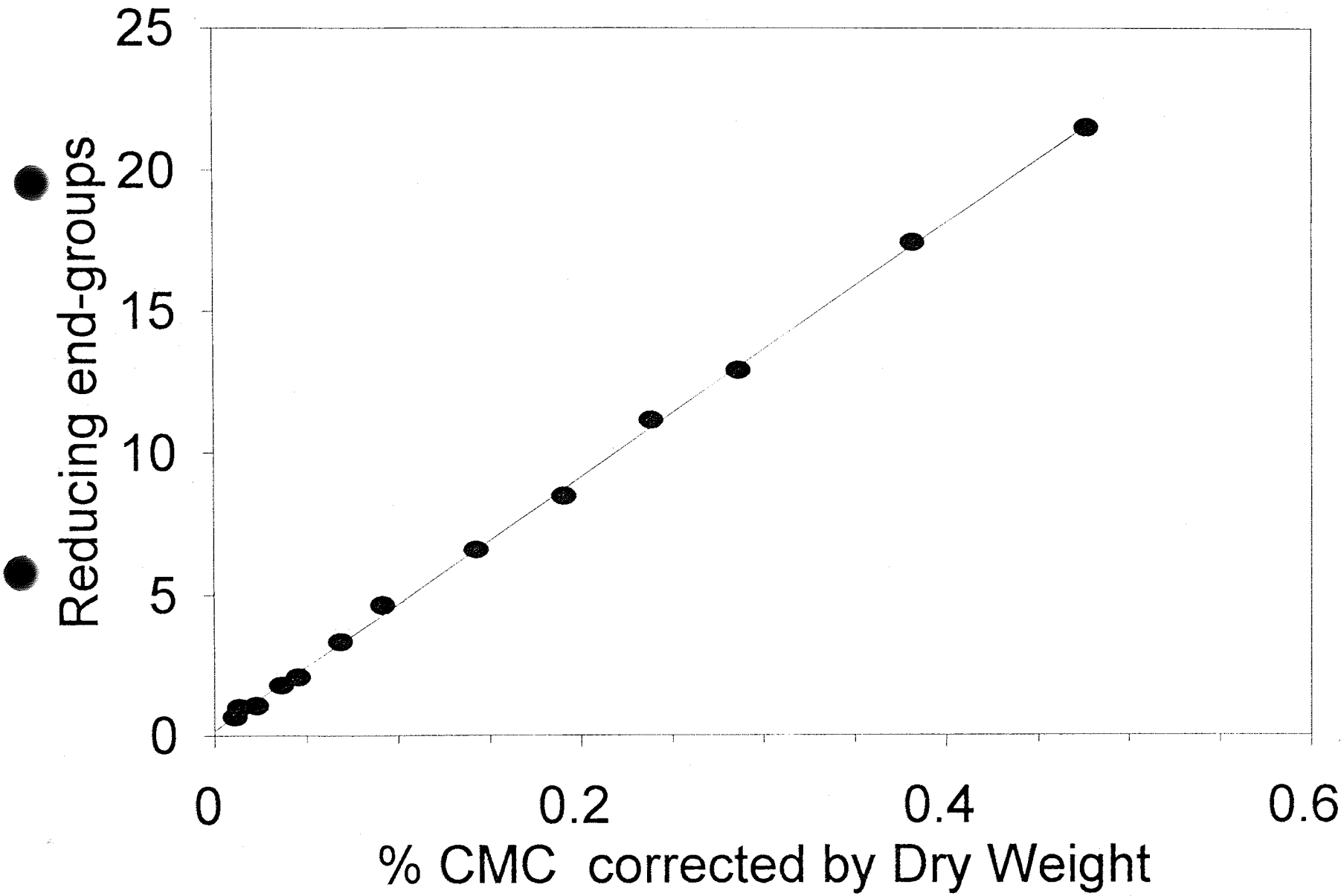
Also, the BCA assay has been proven useful for the relative quantification of CMC in solution. The results of the analysis of reducing end-groups of a series of dilutions (from a single batch) of CMC is shown in Figure 2. This type of determination can be particularly useful in evaluating dilutions of a known (dry weight) batch of CMC.

4.2 Assays with purified enzymes

Four purified endoglucanases were used, namely:

- EGI from *Trichoderma reesei*; assayed at pH 5.0/ 40°C
- EGII from *Trichoderma reesei*; assayed at pH 5.0/ 40°C
- E1 from *Thermomonospora fusca*; assayed at pH 5.5/ 50°C and pH 5.0/ 40°C
- E5 from *Acidothermus cellulolyticus*; assayed at pH 5.8/ 65°C and pH 5.0/ 40°C

Figure 2. Reducing end-group determination of CMC dilutions



T. reesei EGI and EGII were supplied by Genencor International Co. *A. cellulolyticus* and *T. fusca* were kindly provided by Dr. M. Himmel, NREL. All the enzymes were assayed at a final concentration of 10 ng/mL during the incubation with the substrate.

The results of a comparison among the enzymes using the capillary viscometer, the Brookfield and the Carri-Med systems are presented in Figure 3. The three systems give consistent patterns; note the much higher number of experimental points obtained with the Brookfield (1 reading/30 sec) and Carri-Med (1 reading/10 sec) when compared to the capillary viscometer (total of 10 readings during the incubation period). Although the capillary system accommodates fewer points, it is more sensitive, and is recommended.

For comparative purposes, adopting relative measurements, any of the viscometers tested are suitable. For absolute measurement, however, the capillary system is recommended even though it is often more time consuming and requires the estimation of several viscometric constants (see Section 3).

While the viscometric parameters are not as yet completely estimated we continue to express our results in changes in fluidity, as shown in Figure 4. A more linear curve is obtained when plotting the reciprocal of specific viscosity *vs.* time, which is helpful in the calculation of the initial slope of the reaction curve.

The increase in reducing end-groups with time is a direct measure of hydrolytic activity, but does not reflect where along the chain hydrolysis occurs. Combining the data from viscosity reduction, expressed as fluidity, with a corresponding increase in reducing end-groups, gives a more complete picture of endo action. This kind of relationship has been used in the past; however now it is more reliable at initial stages of hydrolysis due to the high sensitivity brought by the use of the BCA assay.

Concomitant to the capillary measurements, aliquots of the CMC solutions were incubated with the endoglucanases to estimate their activity through the formation of reducing end-groups. Figure 5 represents the results of fluidity *vs.* reducing endo-groups. This type of plot gives information on the degree of randomness of the enzymatic attack as correlated to hydrolysis of the internal bonds of the substrate.

Considering the potential application of these endoglucanases in processes such as simultaneous saccharification and fermentation (SSF), we conducted experiments under standard conditions of pH 5.0 and 40°C. The results of fluidity *vs.* reducing end-groups are presented in Figure 6.

The reliability and high sensitivity of the BCA method also allowed its use for the estimative of the K_m and V_{max}. Lineweaver- Burk type plots were used for this purpose, as shown in Figure 7. Preliminary results of K_m and V_{max} for each assayed enzyme tested are presented in Table 2

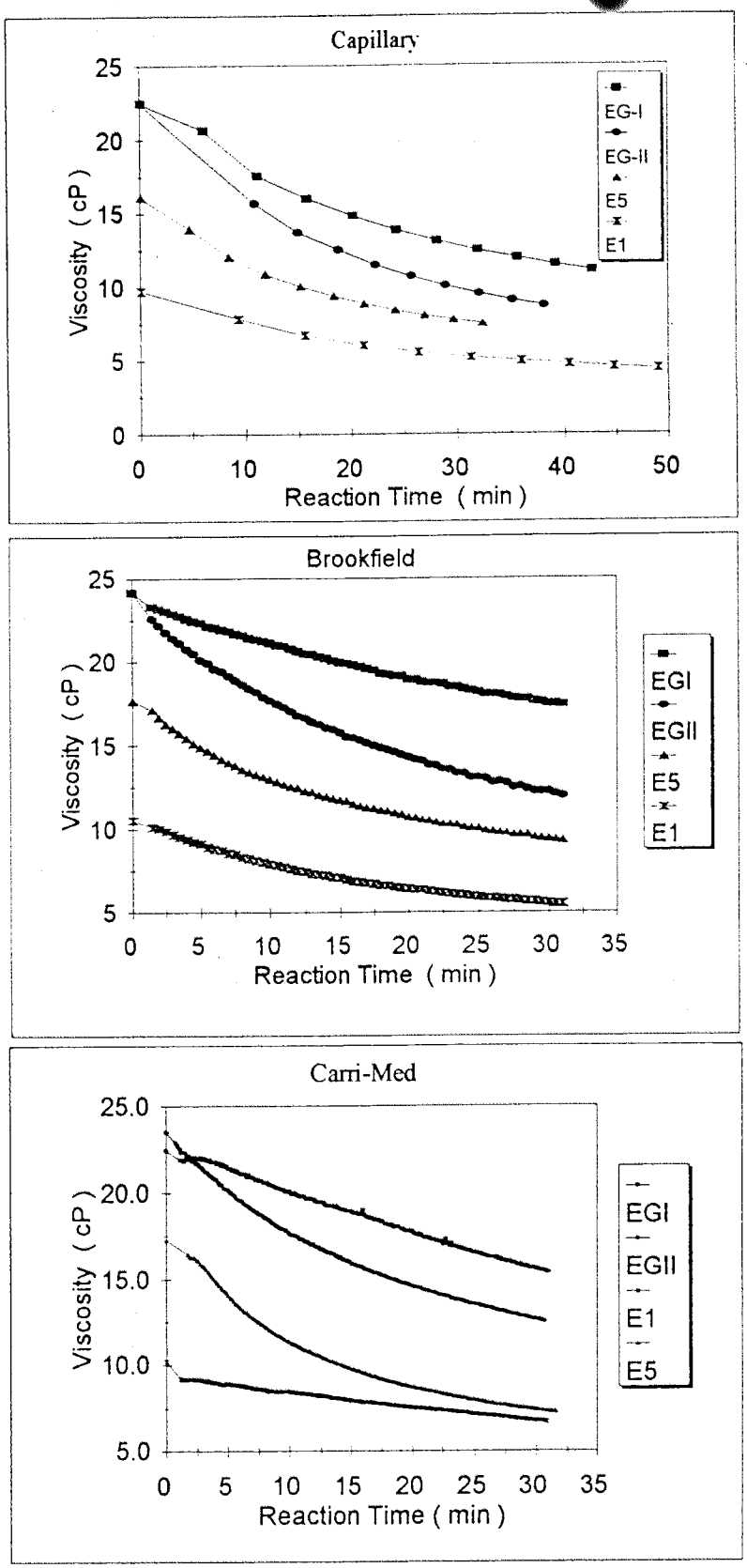


Figure 3. Comparison of rheological Instruments (CMC).

Figure 4. Cellulase activity measured with 0.5 % CMC at the optimal conditions of pH and Temperature (see text for details).

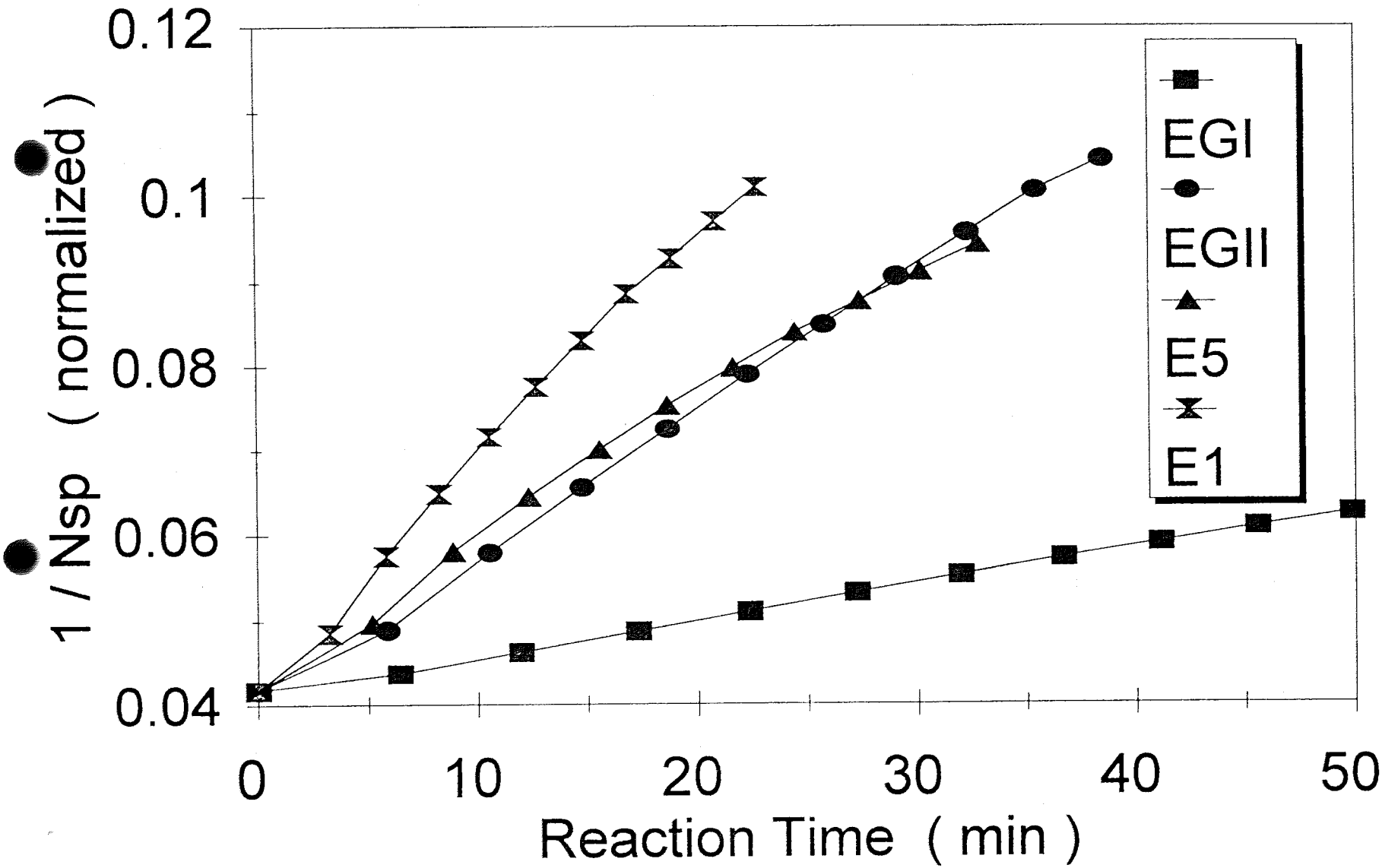


Figure 5. Relationship between fluidity and formation of reducing end-groups by endoglucanases acting on 0.5 % CMC at their optimal conditions.

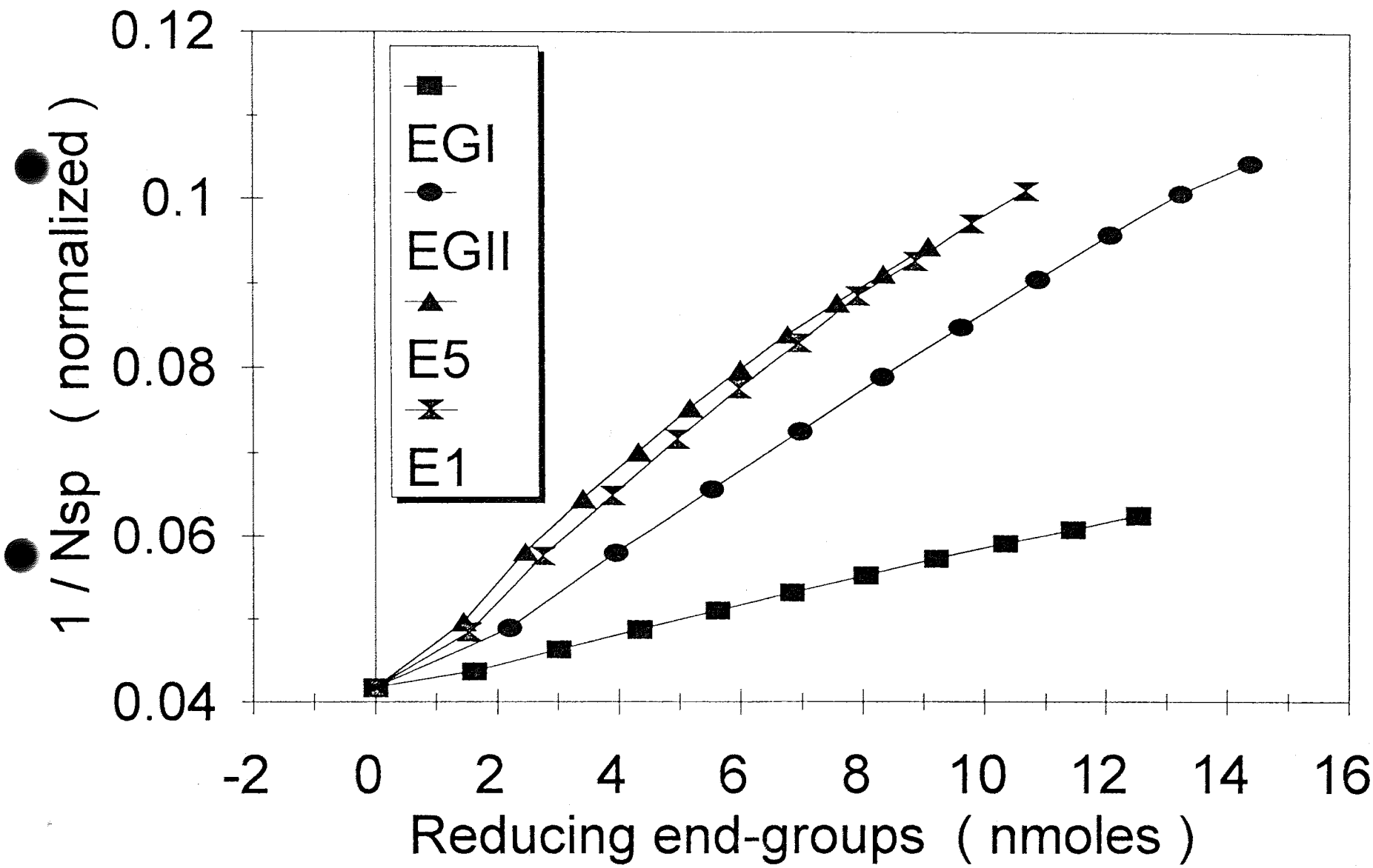
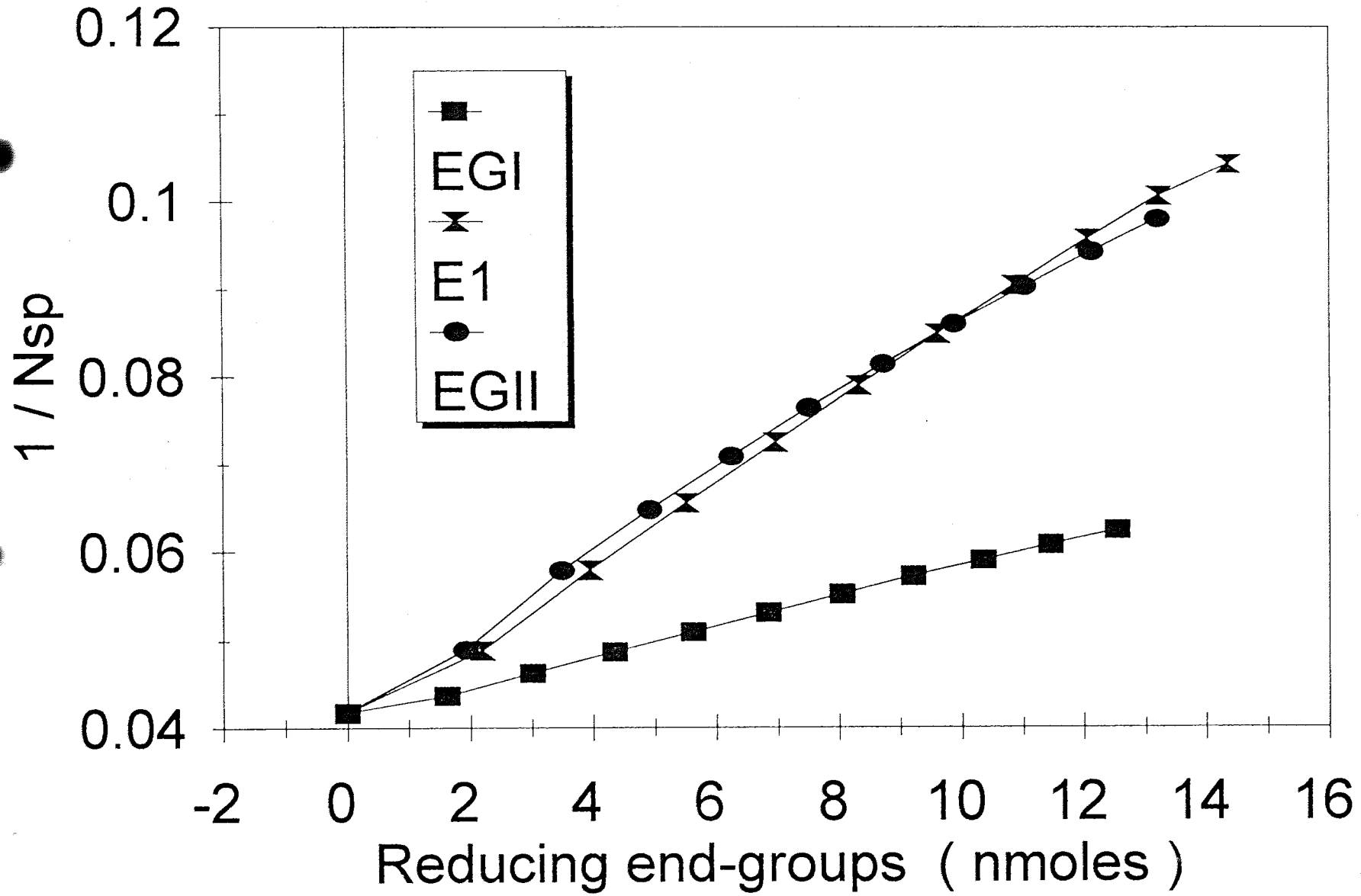
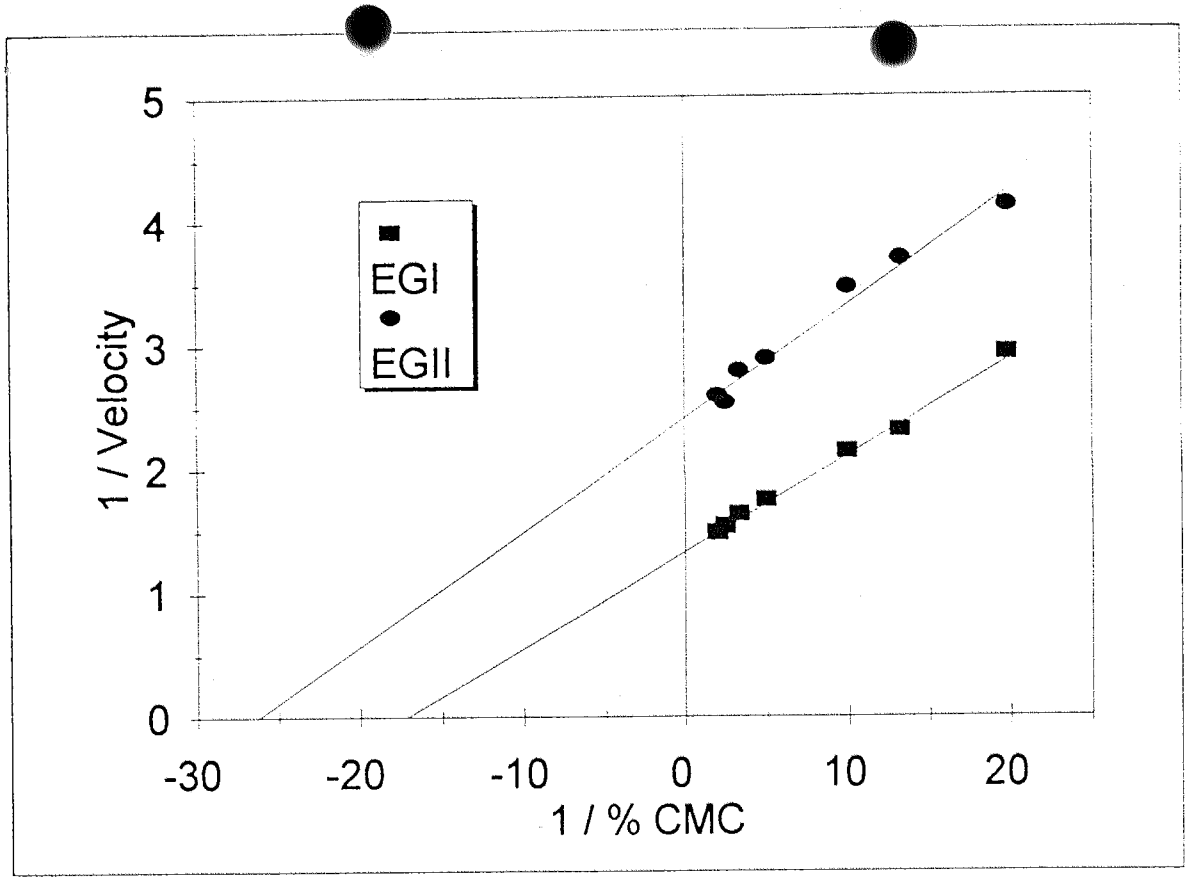


Figure 6. Relationship between fluidity and formation of reducing end-groups by endoglucanases acting on 0.5 % CMC at pH 5.0 and 40°C





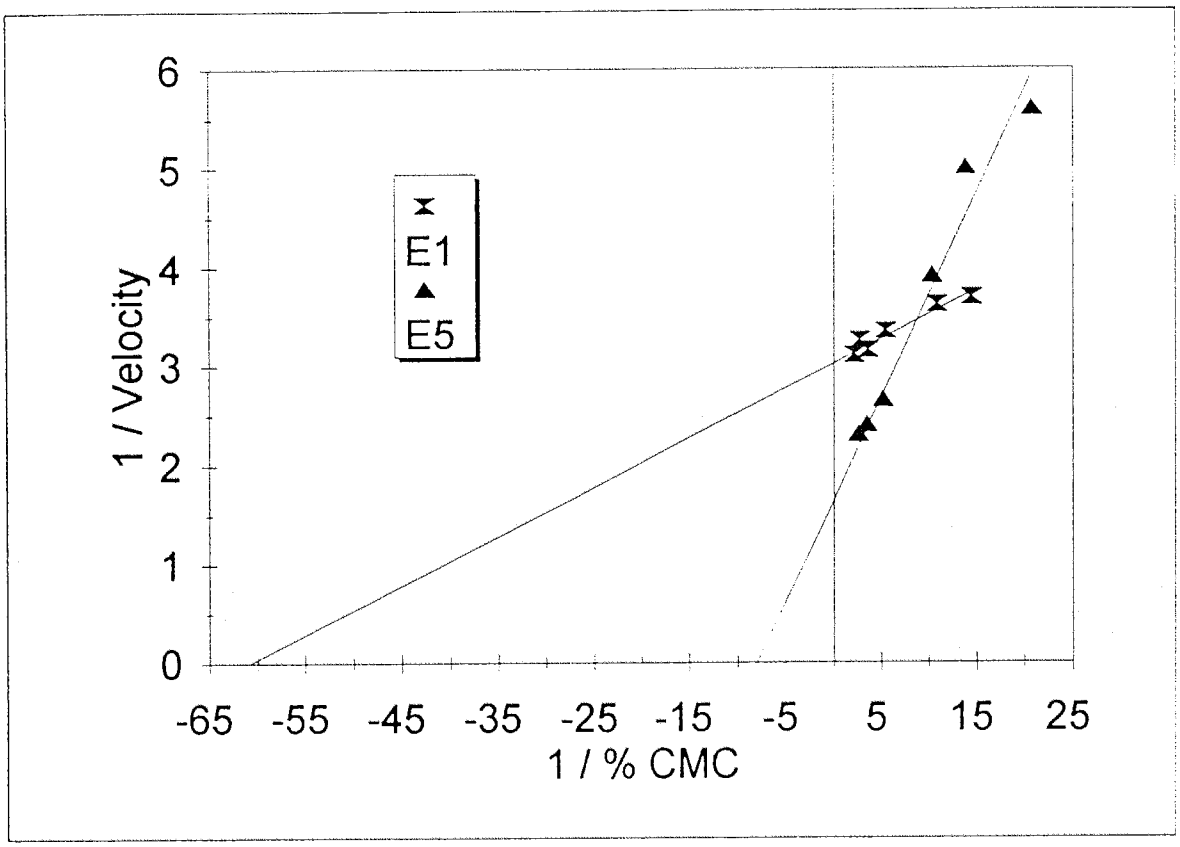


Figure 7. Lineweaver-Burk plots of the BCA data obtained with *T.fusca* E5 and *T.reesei* EGI and EGII and *A.cellulolyticus* E1

Table 2. Preliminary estimated values for Km and Vmax using CMC (DS = 0.89)

Endoglucanase	K m mg. ml^{-1}	V max nM. min^{-1}. ng^{-1}
EGI *T. reesei*	0.58	1.35
EGII *T. Reesei*	0.45	2.89
E1 *A. cellulolyticus*	0.22	3.48
E5 *T. fusca*	1.18	2.22

4.3 Assays with complex substrates

Although concentrated efforts are required for an improvement of the analysis of purified endoglucanases, there is another approach which also deserves attention. In practice, some of these enzymes, or enzymatic systems will be used in saccharification processes of plant biomass. Plant biomass represents a wide diversity of complex substrates, which need to be analytically tested for the evaluation of their susceptibility to enzymatic attack. The problem to be addressed is how to follow the efficiency of the enzymic action when using complex substrates.

Rice straw was selected as a potential lignocellulosic substrate and is locally available. In the Sacramento Valley there is a large production of rice and in recent years the burning of rice straw has been under strict control because of pollution problems.

Two types of experiments were conducted using rice straw incubated with GC 123™ (Genencor Intl.), a commercial preparation containing endoglucanases, exo-cellobiohydrolases and ß-glucosidase. First, the production of total reducing groups and glucose was followed, using the BCA assay and glucose oxidase assay, respectively. Second, an attempt to use a rheological method to follow the cellulose degradation was tried.

Three samples of rice straw (from a single lot) were compared:

- rice straw, coarse particles (sieved through 4 mm screen);
- pretreated rice straw, coarse particles (sieved through 4 mm screen);
- milled rice straw (sieved through 1 mm screen).

The pretreatment used was for delignification of the substrate, and increase the accessibility of the cellulose fraction of rice straw to the cellulases. The pretreatment consisted of treating the rice straw with 1% H_2O_2 at alkaline pH ≥10.5, for 18 hours, followed by washings until neutral pH, filtration and air-drying.

The results of the reducing sugar analysis are presented in Figure 8. The higher susceptibility of the pretreated rice straw to the cellulase action is evident. Regarding the effect of the particle size of the rice straw it becomes clear that at the particle range assayed the size is not a determinant factor in the access of the substrate by the enzymes. However, the removal of non-cellulosic components (such as lignin) led to a significant improvement of the cellulase hydrolysis.

A preliminary comparison of the infrared spectra (Figure 9) of the rice straw substrate before and after the pretreatment does not reveal significant changes. The spectral regions of high interest are those of 1730 cm^{-1} (due to unconjugated carbonyl ketones and carboxyl groups) and 1620 cm^{-1} (due to stretching in *p*-substituted aryl ketones).

Measurements of the viscosities of the rice straw suspensions in steady flow were not possible. The imposition of steady flow on suspensions of pretreated rice straw (5%) caused an aggregation of the straw particles which expelled water. Thus the structure of the suspensions were strongly affected by the application of a steady shear. Another approach to measure the effect of enzymatic hydrolysis on the rheology of rice straw supensions was to apply a small oscillatory shear strain (0.005 amplitude, 1Hz) which would not induce structural changes in the suspensions. The shear stress induced by the strain was measured as the complex modulus $|G^*|$ which was defined as the ratio of the amplitude of the oscillatory stress to strain. A control experiment which measured $|G^*|$ for a 5% suspension over time showed some change in the modulus, Figure 10, but overall the change was small as compared to the change in the modulus when an endoglucanase was added. The enzyme used was GC 123 (Genencor Intl.), and 200 µL of a 131 mg/ml enzyme solution was add to 5g of the 5% rice straw suspension. The magnitude of the modulus $|G^*|$ usually reflects the particle concentration and interactions among the dispersed particles. Since there was no significant change in particle size during this experiment, the reduction in the modulus was attributed to a weakening or reduction in number of particle-particle interactions by the action of endoglucanase.

In addition to rice straw, almond hulls also represent a significant volume of substrate considering that California grows all the almonds produced in the U.S. A preliminary assay using almond hulls as a substrate for cellulases was carried out. As a source of cellulase, GC 123™ (Genencor Intl.) was used. The substrate was previously ground and autoclaved (to eliminate the interference of possible microbial enzymes present). Subsequently, the hulls were treated with cellulase for 5 days. The production of total reducing sugars, as well as glucose (as described for rice straw) was followed. The results are shown in Figure 11.

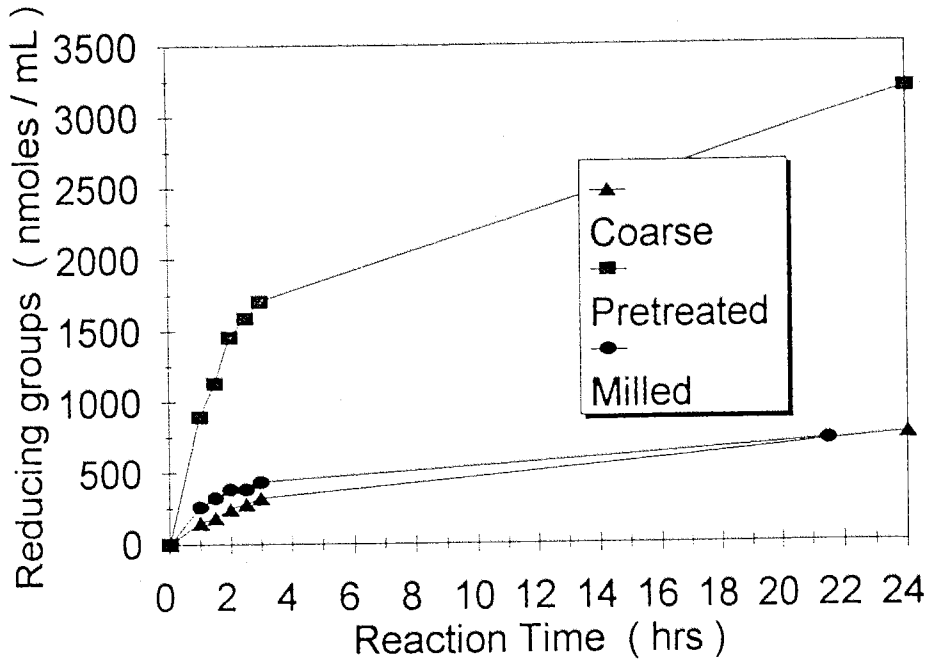


Figure 8A. Total reducing end-groups produced from Rice Straw. (After digestion with GC123 100 µg / mL)

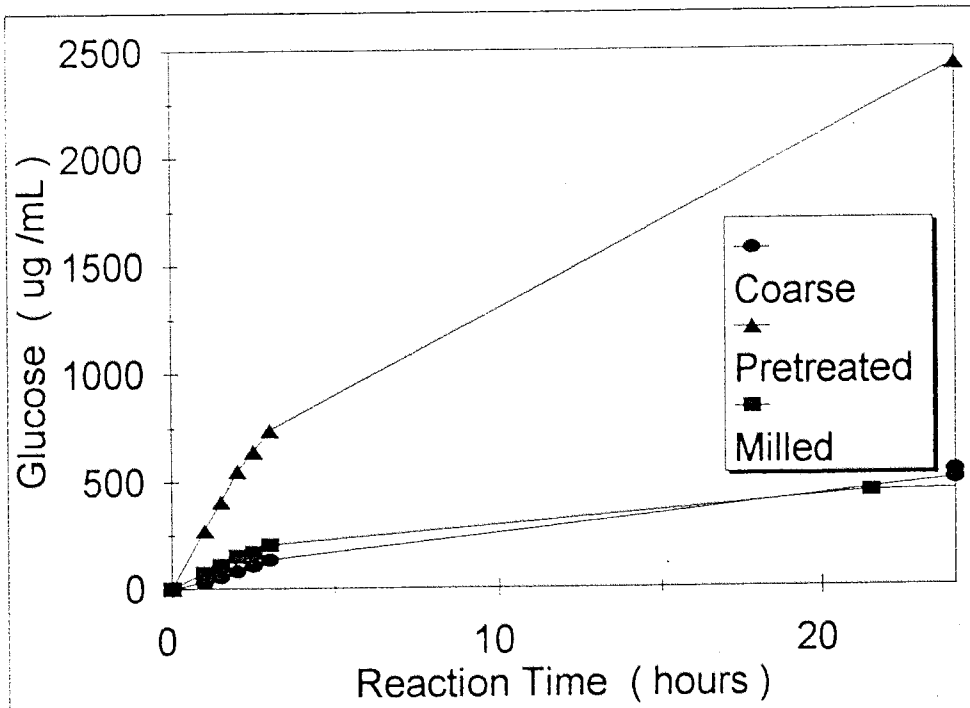


Figure 8B. Glucose produced from Rice Straw. (After digestion with GC123 100 ug / mL)

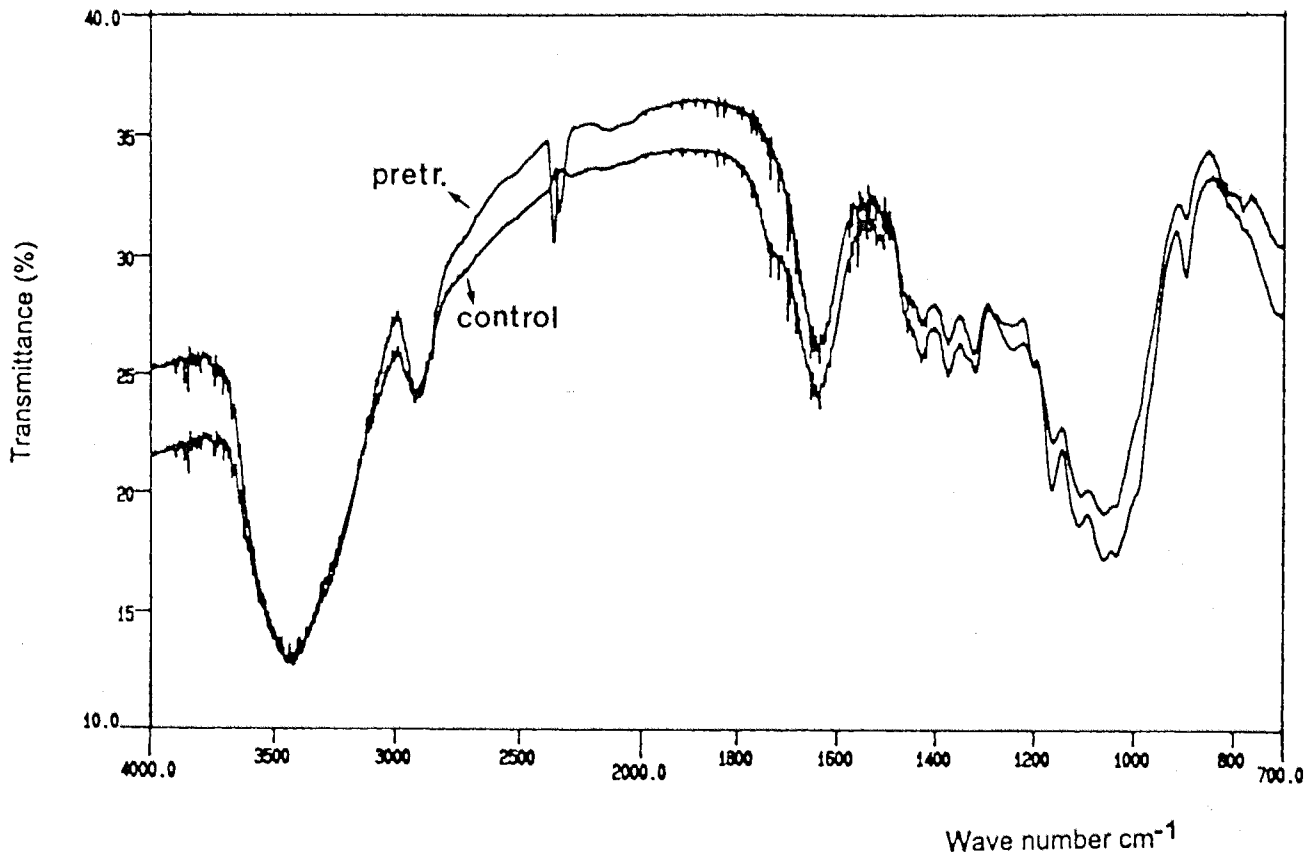


Figure 9. Infrared spectra of rice straw substrate before (control) and after pretreatment (pretr.) with H_2O_2 at alkaline pH.

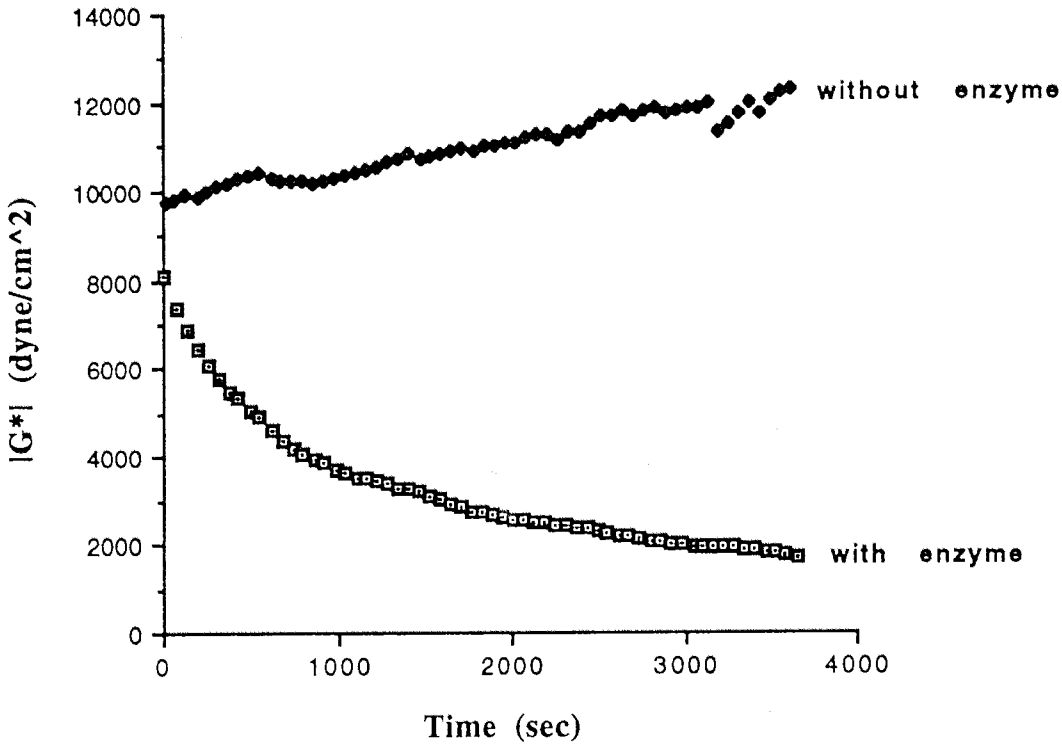
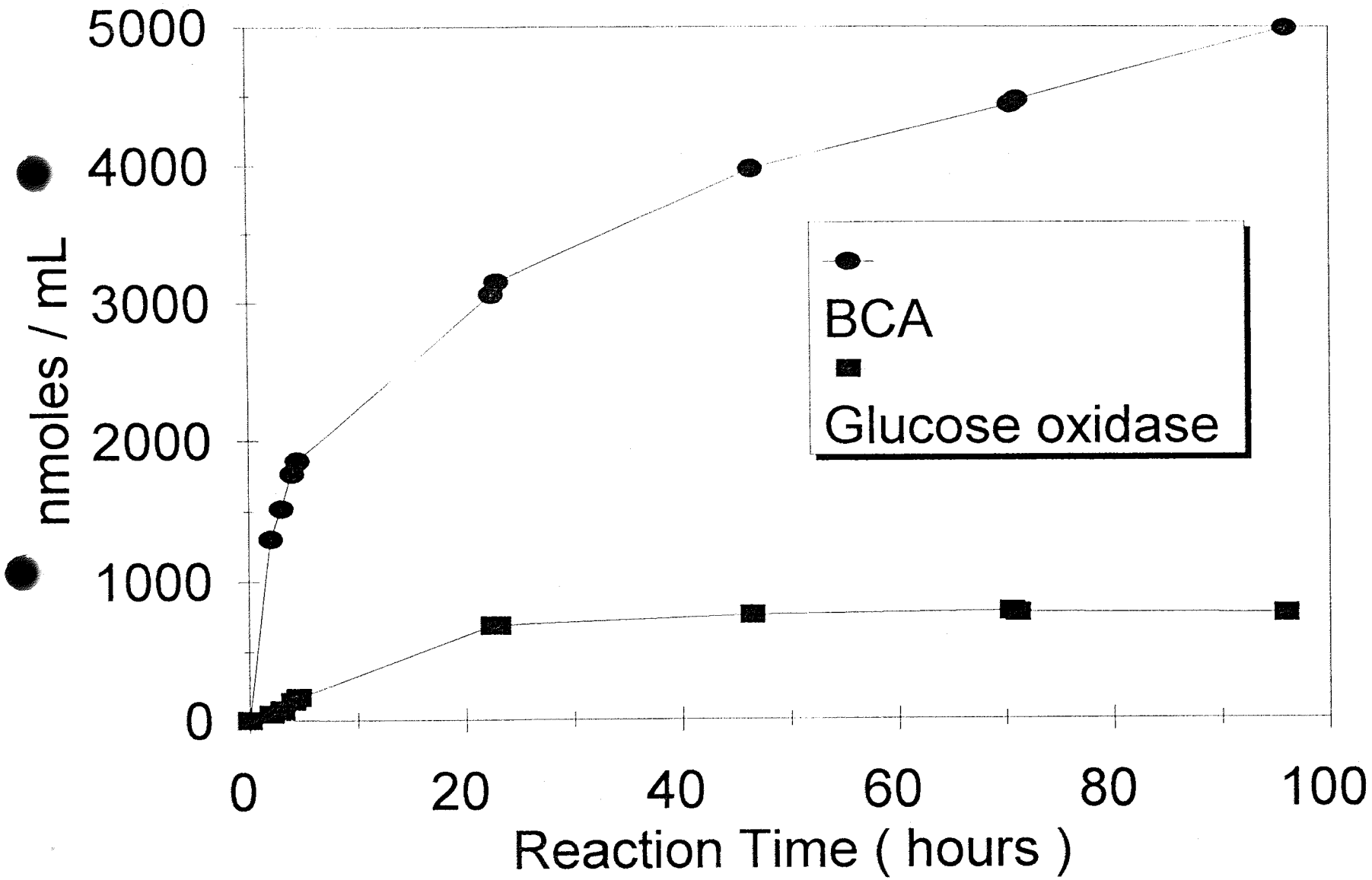


Fig. 10 Change in the complex modulus of a 5% pretreated rice straw suspension, with and without enzyme. The enzyme addition was 200 µl GC123 (131 mg/ml) in 5 g rice straw suspension.

Figure 11. Total reducing end-groups and Glucose produced from Almond Hulls. (After treatment with GC123 100 µg / mL)



5. CONCLUDING REMARKS

In an attempt to establish a relation between the tasks presented under the objectives of this subcontract (Section 2) and the accomplishments, we should mention that:

1. - Based on a literature review it was possible to identify the methods used for endoglucanase assay based on viscometric measurements. A thorough analysis of the relevant papers published on this topic in the last 35 years allowed us to write the report "*State of the Art Report on Viscometric Methods of Analysis of Endoglucanase Activities*", prepared in January 1992.

The experimental evaluation of the existing methods has been carried out throughout the development of this subcontract. Some of the problems, such as instability of the substrate preparation, selection of type of substrate and viscometers were successfully addressed. Some of the theoretical difficulties are being overcome, however this is still a task to be completed. In Section 3 some of the basic problems in adopting the existing methods were discussed. Most of the investigators (Almin and Eriksson, 1967; Werner, 1969; Hulme, 1971; Manning, 1981) gave a more detailed mathematical treatment for the analysis of endoglucanase activity which were based on well established equations. Nevertheless, in some instances the application of these relationships was not very careful. Criticisms of these treatments appeared in some of the original papers above cited.

2. - ***"Recommended Viscosity Assay Method" is the following:***

Preparation of substrate solution
The following procedure is being used for the preparation a stable CMC solution for use in evaluating endoglucanase activity.

> Weigh 2.5 g of CMC 7H3SF (Aqualon Cellulose Gum, supplied by Aqualon Co., Wilmington, DE). It should be noted that CMC should be stored in a dessicator to minimize any moisture adsorption.
>
> Add the cellulose derivative slowly (to avoid formation of lumps) to 500 mL of 0.05 *M* sodium acetate buffer pH 5.0 under vigorous agitation.
>
> After completing the addition, stir the suspension for 1 h at room temperature.
>
> Transfer the suspension to a blender and mix for 1 min at high speed.
>
> Heat the suspension under stirring for 1 h at 80°C.
>
> Filter through sintered glass (coarse) into a vacuum filtering flask.
>
> Degas under low pressure and agitation for 1 h at room temperature.
> Store the solution at 4°C.

We have observed that the viscosity is kept constant for at least 20 days without addition of a preservative.

It is recommended to determine the dry weight of the solution to confirm the exact final concentration of CMC in the preparation. This can be easily done by weighing 25 g of solution, in duplicate, in trays to be transferred to a vacuum oven kept at 60°C, until constant weight. A blank with the buffer should also be dried in order to discount the contribution of the buffer to the dry weight.

Note: This procedure is being further examined to compare the effects of heating and of blending on the substrate and to more broadly compare different types of commercial soluble cellulose derivatives.

Capillary Viscometry

Capillary tube: Ubbelohde type (for example: Schott cat. 545.20, for use with 0.5% CMC at 40°C; it gives drain times > 200 sec).

Procedure: In a wide-mouth plastic tube weight 22 g of 0.5% CMC solution: put the lid on the tube and pre-incubate at 40°C for about 10 min.

For an enzymatic analysis: with a micropipette remove an aliquot of CMC solution identical to the volume of enzyme necessary for the assay.

Add the enzyme solution and start timing the reaction.
Cover the tube. Invert a few times to mix.
With a disposable syringe take 20 mL of the mixture and load immediately the viscometer.
Start the run.

For a Blank analysis: With a micropipette remove an aliquot of CMC solution correspondent to the same volume of enzyme that will be added for the enzymatic assay.

To complete the Blank add an identical volume of buffer of that of CMC removed.
Cover the tube. Invert a few times.
Take 20 mL of the mixture with a syringe.
Load immediately the viscometer.
Start the run.

Note: The aliquot of endoglucanase to be added depends on the enzyme activity. In the example given above, a 220 µL of an enzyme solution containing1µg/mL, when added to 22 mL of substrate gives a final concentration of 10 ng/mL.

3. - Regarding the introduction of new rheological methods, we have started the use of the Carri-Med rheometer dedicated to the analysis of degradation of cellulose, either in pure form (such as bacterial cellulose) or in complex form (such as plant biomass, for example, rice straw).

4. - We have been participating with NREL researchers in the analysis of a few purified endoglucanases. As soon as we complete the recommendation for the

methodology, it is expected to be organized a collaborative study to evaluate its reproducibility.

5. - A preliminary literature compilation of biochemical data on endoglucanases was presented in January 1993 (Appendix 2). We should now consult with NREL for an evaluation of the direction adopted, in order to continue and complete the data-base, or reorient its approach before completion.

6. ACKNOWLEDGMENTS

The authors are grateful to Genencor International for the supply of *T. reesei* EGI and EGII and GC123, and for the loan of a Schott America automated capillary system. Also, it was very appreciated the attention of Dr. David Wilson, Cornell University, in supplying *T. fusca* enzymes and Dr. Michael Himmel, NREL for providing *T. fusca* E5 and *A. cellulolyticus* E1.

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8. Appendices

1. Experimental protocols for the viscometric and reductometric analysis

2. Literature Review and Data-Base of Biochemical and Kinetic Values for 1.4 endo-ß-D-glucanase

Appendix 1. Experimental protocols for the viscometric and reductometric analysis

Capillary Viscometry
Instrument: Schott America automated system (Yonkers, NY)
Capillary tube: Ubbelohde type (Schott cat. 545.20, for use with 0.5% CMC at 40°C; it gives drain times > 200 sec).

Procedure: In a wide-mouth plastic tube weight 22 g of 0.5% CMC solution: put the lid on the tube and pre-incubate at 40°C for about 10 min.

For an enzymatic analysis: with a micropipette remove an aliquot of CMC solution identical to the volume of enzyme necessary for the assay.

Add the enzyme solution and start timing the reaction.
Cover the tube. Invert a few times to homogenize.
With a disposable syringe take 20 mL of the mixture and load immediately the viscometer.
Start the run.

For a Blank analysis: With a micropipette remove an aliquot of CMC solution correspondent to the same volume of enzyme that will be added for the enzymatic assay.

To complete the Blank add an identical volume of buffer of that of CMC removed.
Cover the tube. Invert a few times.
Take 20 mL of the mixture with a syringe.
Load immediately the viscometer.
Start the run.

Rotational Systems
1- Instrument: Brookfield DV-III rheometer (Stoughton, MA) with an adapter (SC4-18/13R) with chamber for small sample volumes.

Procedure: Eight mL of 0.5% CMC solution are weighed into the small chamber, which is then pre-incubated at 40°C for 15 min.

Proceed as described for the analysis with the capillary viscometer removing an aliquot correspondent to the volume of enzyme required for the assay.
After the addition of the enzyme solution (or the buffer) move the chamber up and down for 1 min to mix the sample.
Start the spindle at 60 rpm. After 2 min take the first reading and subsequently a reading each minute thereafter.

2- Instrument: Carri-Med controlled stress rheometer (TA Instruments Co., Valley View, Ohio)

Procedure: Five grams of 0.5% CMC solution are weighed into a tube. The tube is placed into a water bath at 40°C for about 15 min.

Fifty µL of CMC solution are removed from the tube and 50 µL of enzyme solution are added.

Timing of the assay is begun, and the tube is inverted several times to mix.

Approximately 3 mL of sample are loaded onto the Carri-Med Peltier plate and the ram is raised carefully to avoid bubble formation. The procedure is then started and is run for 30 - 45 min.

For the Blank, buffer is used in place of the enzyme solution and the procedure is only run for 2 min.

The Carri-Med settings used are as follow:

- Temperature: 40°C

Cone: 6 cm acrylic, 1:59 degree angle

Gap: 50 µm

Procedure: Flow, controlled stress: 15 dyne/cm^2

Equilibration time: 0, peak hold curve only

BCA Assay

Twenty-five grams of 0.5% CMC solution are pre-incubated in a wide-mouth tube at 40°C.

After 15 min two aliquots of 1 mL (Blanks) are transferred to tubes containing 0.25 mL of alkaline mixture (Na_2CO_3 / $NaHCO_3$, 54.28 g/L and 24.2 g/L, respectively).

A 230 µL aliquot of CMC solution is removed and discarded.

A 230 µL aliquot of endoglucanase solution is added to the CMC solution.

At sequential intervals of 5 min, 1 mL aliquots are removed and transferred to tubes containing 0.25 ml of alkaline mixture to inhibit the enzyme.

Add 0.8 mL of BCA reagent* to each of the assay tubes containing 1 mL of CMC incubation mixture and 0.25 mL of alkaline mixture.

After agitation, the tubes are covered with marbles and heated in a water bath at 80°C for 30 min.

Remove the tubes from the water bath, cool them in running water.

Read the absorbency at 560 nm.

A standard curve with glucose in the range of 10 - 250 µL (stock solution 50 mg/mL) should be prepared simultaneously.

The aliquot of endoglucanase to be added depends on the enzyme activity. In the example given above, a 230 µL of an enzyme solution containing 1 µg/mL, when added to 23 mL of substrate gives a final concentration of 10 ng/mL.

* The BCA reagent is freshly prepared by mixing equal volumes of solutions A and B:
Solution A: 54.28 g/L Na_2CO_3, 24.2 g/L $NaHCO_3$, 1.942 g/L disodium 2.2' bicinchoninate. Solution A shout be kept in amber bottle.
Solution B: 1.248 g/L $CuSO_4.5H_2O$ and 1.262 g/L L-serine. Solution B should be kept in amber bottle under refrigeration.

Literature Review and Data-Base of Biochemical and Kinetic Values for 1,4 endo-β-D-glucanase

1. INTRODUCTION

The multi-enzyme cellulase system[(1)] is composed of:

- endoglucanases (endo-1,4-β-D-glucan-glucanohydrolases, EC 3.2.1.4);
- exo-cellobiohydrolases (1,4-β-D-glucan-cellobiohydrolases, EC 3.2.1.91);
- β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21).

The first group is constituted of internal bond splitting enzymes, while the second and third groups is formed of endwise splitting enzymes. A system containing these three types of enzymes is often referred to as a "true cellulollytic" or a "complete" system.

All cellulases attack the same chemical bond, the β-1,4-glucosidic linkage, nevertheless, in the natural substrates there is a variation in the microenvironment of these bonds (Knowles et al., 1987). These differences can be related to variations in crystallinity, due to hydrogen bonding between chains, and/or the association of cellulose with hemicellulose and lignin.

The observed multiplicity of components is typical in cellulolytic systems; often, five or more enzymes make up a system. However, some systems seem to be much more complex. It was reported that *Clostridium* may produce 21 different endoglucanases (Teeri et al., 1983). Synergism between components and apparently overlapping specificities of the cellulolytic enzymes indicate that the specificity of many of these enzymes has not been unequivocally demonstrated (Knowles et al., 1987).

Most of the fungal cellulases in culture filtrates are glycoproteins (Coughlan, 1985). The carbohydrate moiety may confer stability to the secreted cellulases.

Bacterial cellulases are, mainly, cell-wall bound, while only fungi appear to excrete large amounts of active forms of cellulases into the culture media. Among the bacteria studied, there are a few notable exceptions, such as *Clostridium thermocellum*, which seems to liberate an extracellular enzymatic system (Wood, 1985, Wood, 1991). An overview of the best studied endoglucanases produced by cellulolytic microorganisms is presented in section 2.

(1) Coughlan and Ljungdahl (1988) recommended the use of the term "cellulase system" to be used instead of "cellulase complex", unless it is demonstrated that the cellulases in question are part of a complex.

In the assessment of cellulolytic activity, the choice of substrate is of major concern. Sometimes native cellulose is considered an unsuitable substrate, because of its insolubility and low rate of hydrolysis, and also because some cellulases do not attack crystalline substrates. If the objective is the identification of different types of cellulase activity, alternative substrates should be used. This topic is briefly discussed in some more detail in Section 3.

2. *CELLULASES AND BIOCONVERSION OF CELLULOSE IN NATURE*

Most of the cellulose degradation occurring in nature is carried out by microbial enzymes produced by a variety of microorganisms, including fungi and bacteria. Ljungdahl and Eriksson (1985) stated that around 90% of the cellulose (biomass turnover) is degraded by aerobic microorganisms, while 10% is converted by anaerobic microorganisms.

Microorganisms that degrade cellulose are abundant and ubiquitous in nature. However, in some cases the products of growth are microbial cells and metabolic products, such as carbon dioxide and methane. Nevertheless, a few fungi and bacteria are known for their ability to produce highly active cellulases capable of extensive degradation of insoluble cellulose to soluble sugars (Bisaria and Ghose, 1981). Brown-rot fungi degrade cellulose even faster than they utilize the products (Blanchette et al., 1978). Nevertheless, in nature, the microorganisms (aerobic or anaerobic) engaged in cellulose degradation act in consortium with other non-cellulolytic species, which ensures a substantial greater rate and extent of cellulose conversion than in monoculture (Coughlan, 1990).

According to Saddler (1986) cellulolytic activity of fungi is generally 50-1000 times higher than in the most active bacteria, due to the higher secretion of extracellular enzymes by fungi. In bacteria, cellulolytic enzymes are commonly produced in reduced amounts (less than 0.1 g/L), while fungal cellulases are available in larger amounts (more than 20 g/L) (Knowles et al., 1987).

It has been repeatedly emphasized in the literature (Mandels, 1975) that, although many fungi can grow on cellulose or produce cellulolytic enzymes that degrade amorphous cellulose, there are only a few which produce the "complete" extracellular system to extensively degrade crystalline cellulose *in vitro*. Some microorganisms may grow faster than fungi on diverse forms of cellulose, such as the following bacteria: *Thermomonospora sp., Bacteroides succinogenes, Clostridium thermocellum* and *Acetivibrio cellulolyticus*. However, these bacteria have not demonstrated extracellular cellulase activity comparable to that from *Trichoderma reesei* (Johnson et al. 1982). The thermophilic bacterium *Clostridium thermocellum* produces an extracellular

cellulase complex (cellulosome) highly active on crystalline cellulose (Wu, 1993). Johnson et al. (1982) described a cell-free preparation of *Clostridium thermocellum* with cellulase activity as active as *Trichoderma reesei* cellulase. However, most preparations of *Clostridium thermocellum* do not exhibit this property (Wood, 1985).

2.1 Cellulolytic Organisms

Different environments have been investigated in the search for promising sources of cellulases. The discovery of *Acidothermus cellulolyticus genus novum, species novum* (Mohagheghi et al., 1986) proved that hitherto unknown potential cellulolytic microflora remain to be discovered.

Cellulolytic organisms have been searched for in nature, such as decaying wood, dead plant parts, pathogens in plants, soil, among others. Some of the most active cellulase systems known, such as white-rot fungi, brown-rot fungi, and soft-rot fungi, were isolated from those types of material.

Enary (1983) classified the microbial cellulases according to their localization in two groups:
- Cell-bound, intracellular, surface-bound;
- Extracellular.

Among fungi the ability to produce extracellular cellulases in widespread, while most bacterial cellulases are cell-bound. According to Zeikus (1980) the best producers of extracellular cellulolytic enzymes are amongst the genus *Clostridium*..

Some bacteria do not excrete large amounts of cellulases but are of interest because of their ability to grow on cellulosic substrates (Enary, 1983). In spite of secreting low amounts of cellulases, several anaerobic bacteria exhibit a quite efficient degradation of crystalline cellulose. The best studied example is *Clostridium thermocellum*..

In order to find organisms producing more thermostable enzymes, thermophilic organisms have also been studied. However, it should be pointed out that cellulases from thermophilic organisms are not necessarily more heat-stable than cellulases from mesophiles (Mandels, 1975). It is worth mentioning that *Acidothermus cellulolyticus* cellulases possess the highest temperature tolerance reported to date (Tucker at al., 1989).

Coughlan (1985) listed some cellulolytic organisms of established or portential commercial use:

Fungi

Agaricus bisporus
Aspergillus fumigatus
Aspergillus niger
Aspergillus terreus
Botryodiplodia theobromae
Chaetomium thermophillum
Eupenicillium javenicum
Fusarium solani
Humicola insolens
Macrophomina phaseolina
Myrothecium verrucaria
Myceliophthora thermophila
Pellicularia filamentosa
Penicillium citrinum
Penicillium funiculosum
Penicilllium irensis
Penicillium janthinellum
Penicillium variabila
Pestalotiopsis westerdijkii
Polyporus adustus
Polyporus tulipiferae (Irpex lacteus)
Polyporus versicolor
Poria spp.
Sporotrichum cellulophilum
Sporotrichum dimorphosporum
Sporotrichum pulverulentum (Chrysosporium lignorum)
Sporotrichum pruinosum
Sporotrichum thermophile
Talaromyces emersonii
Thermoascus aurantiacus
Thielavia terrestris
Tranetes sanguinea
Trichoderma harzianum
Trichoderma koningii
Trichoderma lignorum
Trichoderma longibrachiatum
Trichoderma pseudo-koningii
Trichoderma reesei
Trichosporon cutaneum
Trichosporon pullulans

Bacteria

Acetivibrio cellulolyticus
Bacteroides cellulosolvens
Bacteroides succinogenes
Cellulomonas spp.
Cellvibrio fulvus
Cellvibrio gilvus
Cellvibrio vulgaris
Clostridium thermocellulaseum
Clostridum thermocellum
Clostridium thermomonospora
Pseudomonas fluorescens
Ruminococcus albus

Actinomycetes

Streptomyces griseus
Thermoactinomycete spp.
Thermomonospora fusca
Thermomonospora curvata

2.1.1 Aerobic Cellulolytic Fungi

Cellulolytic fungi play a major role in the conversion of the biomass of plant origin into soil (Ghosh and Ghosh, 1992). In spite of the fact that a variety of organisms have been tested for cellulase (activity, yield, conditions of production, etc), different species of *Trichoderma* are the most widely used organisms. It was suggested that, by far, *Trichoderma* are the best cellulase producers (Eveleigh, 1987). There are reports showing that, under favorable conditions, in many strains of *Trichoderma reesei* the cellulase secretion may reach 90% of the net synthesis of proteins, without any significant autolysis (Ghosh et al., 1984). Besides *Trichoderma*, many species of fungi are notable in their capacities of synthesizing and releasing cellulases into the culture media; these include: *Penicillium funiculosum, Sporotrichum pulverulentum, Fusarium solani, Talaromyces emersonii* (Wood et al., 1988).

Many cellulolytic systems present quite a diversity in composition. It was suggested that the diversity of fungal endoglucanases could be, in part, due to post-secretional proteolytic processing, which could regulate and adjust the substrate specificity of the cellulases (Gong et al., 1977). This type of processing would contribute to the enzyme diversity without increasing the number of genes (Knowles et al., 1987). The multiplicity of components could also be determined by differential glycosylation. It seems that both possibilities are operative (Wood, 1985). Some authors argue that different forms of enzymes are genetically determined (Labudova and Farkas, 1983, Bhat and Wood, 1989). Also, antiserum prepared with purified EGI from *Penicillium pinophilum* (Bhat et al., 1989) reacted only with EG I, the same was found with EGII, which reacted only with antiserum prepared with purified EG II. This observation is in contrast with the conclusions of Niku-Paavola et al. (1985) that EGs from *Trichoderma reesei* (strain VTT-D-80133) existed as a series of immunologically related components, with a common original ancestoral gene. Also, specific antibodies raised against purified EGs isolated from *Sclerotinia sclerotiorum* showed no cross-reaction with EGs different than those utilized to prepare the antiserum. Moreover, the sequencing of the N-terminal part of the EGs from *S. sclerotiorum* suggests that these enzymes do not originate from a common polypeptide, and have different genetic origins (Waksman, 1991). It was also suggested that the multiplicity of cellulases could result from aggregation of enzymes with each other and with impurities in culture, or even from manipulation during the purification steps, resulting in artifacts (Wood, 1991).

2.1.2 Ruminal Cellulolytic Organisms

Another ecosystem where microorganisms which degrade cellulose are found is the rumen, a highly anaerobic environment (Weimer, 1992). The ability of ruminant animals to digest fibrous materials results from a fermentation mediated by a complex microflora composed of bacteria, fungi and protozoa. Among these organisms, bacteria represent the major group of fiber-digesting microorganisms in the rumen (Malburg et al., 1992). A list of the ruminal cellulolytic microorganisms is presented in Table 1.

There is an extensive knowledge of cellulases produced by aerobic fungi, while little is known about anaerobic fungi, because their study is more recent. Nevertheless, most of the anaerobic fungi known are able to degrade crystalline cellulose, in comparison with only a few aerobic fungi (Wood, 1992).

It seems that the anaerobic cellulolytic fungi living in the rumen burrow into the fiber, while the rumen cellulolytic bacteria seem to attack the fibers by surface erosion, which could be a complementary role in the hydrolysis of cellulose (Wood et al., 1988). The extracellular cellulolytic enzymes from ruminal fungi, for the most part, resemble those from nonruminal fungi (Weimer, 1992). Ruminal fungi synthesize very active cellulolytic enzymes on a specific basis, however, the amount of secreted enzymes is relatively small. The most extensively studied anaerobic fungi isolated from the rumen is *Neocallimastix frontalis*..

Neocallimastix frontalis strain RK21, when grown in co-culture with the methanogen *Methanobrevibacter smithii*, produces the extracellular endoglucanase, β-glucosidase, capable of a remarkable degree of solubilization of crystalline cellulose, much faster than the cellulase system from *T. reesei* C-30 (Wood et al., 1988), as presented in Figure 1. The authors reported the isolation of a cellulase complex (MW 1-2 million) that was impossible to dissociate. Li and Calza (1991) recently reported the fractionation of 7 endoglucanases from *Neocallimastix frontalis* EB188. Wilson and Wood (1992) found two peaks with CMCase and _-glucosidase activities associated with a high MW and a low MW complexes, extracted from culture filtrates of *N. frontalis* RK21. The authors suggested that cellulases of *N. frontalis* may be cell-bound, or at least not completely cell-free. They raised the possibility of the participation of chitin in maintaining the integrity of the high MW complex.

TABLE 1
Cellulolytic Microorganisms of the Rumen[a]

Bacteria	Protozoa[d]
Predominant species[b]	*Diplodinium pentacanthum*
Fibrobacter succinogenes	*Enoploplastron caudatum*
Ruminococcus flavefaciens	*Epidinium caudatum*
R. albus	*Entodinium caudatum*
	Eudiplodinium bovis
Less predominant species[c]	*E. magii*
Butyrivibrio fibrisolvens	*Ophryoscolex caudatus*
Clostridium longisporum	*Ophryoscolex tricoronatus*
C. lochheadii	*Ostracodinium dilobum*
C. chartatabidium	*O. gracile*
C. polysaccharolyticum	*Polyplastron multivesiculatum*
Eubacterium cellulosolvens	
Micromonospora ruminantium	Fungi
	Anaeromyces mucronatus
	Caecomyces communis
	Neocallimastix frontalis
	N. joyonii
	N. patriciarum
	Orpinomyces bovis
	Piromyces communis
	Ruminomyces elegans

[b] Species that are both present in large numbers in the rumen of most animals examined and that usually degrade cellulose relatively rapidly in pure culture.
[c] Species that are only rarely found in large numbers in the rumen. The rate of cellulose digestion *in vitro* is variable, but generally below those of the predominant species listed above.
[d] Species that have been reported to degrade cellulose.

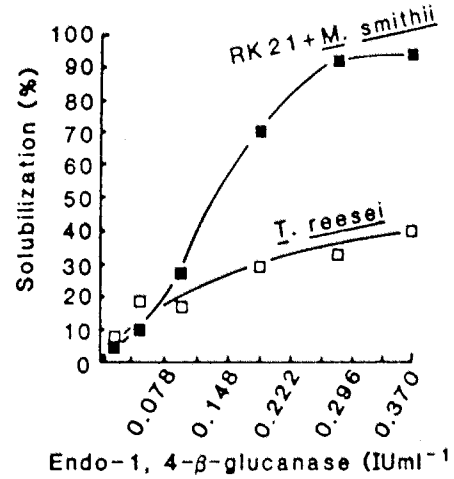

Fig. 1 *Comparison of the cotton-solubilizing activity of the extracellular cellulases of* T. reesei *C30 and* N. frontalis *RK21 - methanogen co-culture. The enzyme-cotton digests were incubated at 37°C (RK21) and 50°C (*T. reesei*) for 72 h. The digests were equivalent in terms of endoglucanase and β-glucosidase.* T. reesei *cellulase was deficient in β-glucosidase activity and was supplemented with β-glucosidase from* F. solani *(4).*

The major cellulolytic organisms in the rumen are the bacteria. Among them the predominate *Fibrobacter succinogenes* (formerly *Bacteroides succinogenes*), *Ruminococcus flavefaciens* and *R. albus*. *Fibrobacter succinogenes* appears to be the most actively cellulolytic ruminal bacterium (Weimer, 1992).

2.1.3 Cellulolytic Bacteria

Despite the production of small amounts of cellulase, when compared to fungal systems, purified bactrial cellulases have been shown to posssess specific activities equal to or surpassing those from fungi (Robson and Chambliss, 1989).

Many filamentous soil bacteria, both mesophilic and facultative thermophilic are included in the order Actinomycetales. These are filamentous aerobes which seem to be major degraders of cellulose in heated residues, such as compost heaps, rotting hay, manure piles and paper mill waste. Among the thermophilic species, *Microbispora bispora* and *Thermomonospora fusca* have been extensively studied. *Thermomonospora* strains excrete cellulases (Wilson, 1992).

Bacterial cellulases, with a few exceptions, are cell-wall bound enzymes (Wood, 1991). Among the bacterial cellulases that do not form stable high molecular mass complexes are: *Cellulomonas, Thermomonospora fusca, Microbispora bispora* and *Clostridium stercorarium*. It seems that these cellulase systems resemble the systems of aerobic fungi (Béguin, 1992).

The most studied anaerobic bacterium, *Clostridium thermocellum*, produces cellulases in the form of tight multienzyme complexes named "cellulosomes", which are difficult to dissociate without loss of activity of the individual components (Lamed et al., 1983).

Compared to aerobic fungi, it is clear that anaerobic bacteria have a different system for cellulose degradation. In fungi, it is well accepted that the synergistic action of EGs, CBHs and β-Gs is involved in the degradation of crystalline cellulose. The enzymes act in a strong synergy in which the final cooperative action is larger than the sum of the individual enzymes action. Nevertheless, the role of each component and how the synergism results in solubilization of the crystalline cellulose is not clearly understood. Commonly, the cellulolytic systems of anaerobic bacteria are constituted of cellulase complexes. These complexes (referred to as cellulosomes, in the case of *Clostridium thermocellum*) adhere to the cellulose to effect its hydrolysis. As the culture ages, the complexes are released from the bacterial cell and from the substrate. For some time the complexes

may be functional while in the culture fluid, but they ultimately decompose to free polypeptides (Coughlan and Ljundahl, 1988).

2.2 Fungal and Bacterial Endoglucanases

Endoglucanases (EGs) are thought to degrade, in a random fashion, amorphous celluloses, cellulose derivatives (CMC, HEC, etc), barley β-glucan and soluble cello-oligosaccharides. The action of EGs on cellulose chains cause a rapid decrease in the viscosity with a low increase in reducing groups (Wood and McCrae, 1979). These enzymes have little apparent ability to degrade crystalline cellulose (Wood, 1992). Due to the common use of CMC as a substrate in the assessment of EG activity, these enzymes are often called CM-cellulases (CMCases).

Multiplicity of EGs is also a rule in most of the organisms investigated so far. Regarding their catalytic properties, when assayed by viscometry and end group analysis, generally each EG gives a characteristic slope of fluidity *versus* reducing groups. There is still uncertainty on the nature and origin of the heterogeneity of cellulases . It is believed that at least some of the heterogeneity is artifactual (Wood, 1991).

It has been difficult to compare results obtained in different laboratories on the various endoglucanases. In many cases, different types of preparations were used, or different sources of cellulases, or different substrates, or even different methodology to assess the cellulolytic activity. It would be most appropriate to use standardized methods, in order to facilitate a comparison of the results.

Some physicochemical characteristics of isolated fungal and bacterial endoglucanases are listed in Table 2.

Some properties of the cellulase complexes produced by some anaerobic bacteria are summarized in Table 3.

Table 2. Physicochemical characteristics of isolated fungal and bacterial endoglucanases

Source	Enzyme	M.W. (kDA)	Carbohydrate content (%)	Isoelectric point	Optimum pH	Optimum temperature (°C)	Km	V_{max}	References
				Fungal Endoglucanases					
Trichoderma reesei* L27	EG I EG II EG III	54.0 (SDS)		4.7 5.5 7.4					Shoemaker et al. (1983)
T. reesi	A_3 (major EG)	25.8 (SDS)		7.3	5.0 (CMC)	55 - 60 (CMC)			Masslot (1992)
T. reesei	EG-III	49.8 (SDS)	15	5.5-5.6	4.0-5.5 (G_3)				Saloheimo et al. (1988)
T. reesei QM 9414	EG 1 EG 2	34.0 (LS) 44.7 (LS)	15.5 12.2						Odegaard et al. (1984)
T. reesei QM 9414	Endo I Endo II	55.0 (SDS) 48.0 (SDS)	10.0 6.5	4.5 5.5					Bhikhabhai et al. (1984)
Trichoderma viride*	I II	12.5 (GF) 50.0 (SE)	21 12	4.60 3.39					Berghem et al. (1976)
T. viride	 II III IV	(SE) 37.2 52.0 49.5	 3,2 14.0 15.9		 3.0-4.0 4.0-4.5 4.0-4.5		G_2 G_3 G_4 1.03(1) 0.35 -- 162 2.65 1.33 1.26 0.28 2.13	G_2 G_3 G_4 0.50(1) 1.22 -- 3.65 24.46 6.7 0.30 0.96 14.1	Shoemaker and Brown (1978a,b)
T. viride	II-A II-B	30.0 (GF) 43.0 (GF)	12 14		4.5-5.0 4.5-5.0	60 50			Okada (1975)

* T. reesei was originally identified as T. viride, but in 1977 it was recognized as a new species (Senecal et al. 1992)

Table 2. Physicochemical characteristics of isolated fungal and bacterial endoglucanases

Source	Enzyme	M.W. (kDA)	Carbohydrate content (%)	Isoelectric point	Optimum pH	Optimum temperature (°C)	Km	V_{max}	References
T. viride	III	45.0 (GF)			4.5-5.0	50	2.94 mM (G_3) 0.67 mM (G_4) 0.56 mM (G_5) 0.45 mM (G_6) 0.054 mM (CMC)	$A_{660\ nm}$/min X 10^{-2} 8.6 14.8 44.3 55.3 18.7	Okada (1976)
T. viride QM 9414	low MW EG high MW EG	20.2 (GF) 51.0 (GF)	0 0	7.52 4.66					[a]Hakansson et al (1978) [b]Hakansson et al (1979)
Trichoderma koningii	E_1 E_{3a} E_{3b} E_4	13.0 (GF) 48.0 (GF) 48.0 (GF) 31.0 (GF)		4.72 4.32 4.32 5.09					Wood and McCrae (1978)
T. koningii					5.5	60	2.5 mg . mL^{-1} (CMC)	0.88 (2)	Halliwell and Vincent (1981)
Aspergillus fumigatus		12.5 (SDS)	0.0	7.1	4.8	60			Parry et al (1983)
Talaromyces emersonii	I II III IV	35.0 (SDS) 35.0 " 35.0 " 35.0 "	27.7 29.0 44.7 50.8	3.19 3.08 2.93-3.0 2.86	5.5-5.8 5.5-5.8 5.5-5.8	75-80 75-80 75-80	K' (3) 1.85 1.68 1.47 1.54		Moloney et al. (1985)
Fusarium solani	1 2 3	37.0 (SDS) 37.0 (SDS) 37.0 (SDS)		4.75 4.80-4.85 5.15					Wood (1971)
Fusarium IInI		28.0 (SDS)	2.9	8.3	4.5-5.5	55	11.6 mg/mL (CMC)		Vaidya et al. (1984)
Sclerotium rolfsii	A B C	50.0 (GF) 27.5 (GF) 78.0 (GF)	+ + +	4.55 4.20 4.51	4.0 2.8-3.0 4.0	74 50 50		G_6 G_{37} CMC 23.1 (4) 34.1 28.3 17.4 19.8 29.1 15.3 17.4 24.1	Sadana et al. (1984)

Table 2. Physicochemical characteristics of isolated fungal and bacterial endoglucanases

Source	Enzyme	M.W. (kDA)	Carbohydrate content (%)	Isoelectric point	Optimum pH	Optimum temperature (°C)	Km	V_{max}	References
Penicillium pinophilum (8 EGs were detected)		SDS GF					(with CMC)	µMol/min	Bhat et al. (1989)
	EG-I	25.0 10.5	+	7.4	4.0-5.0	50-55	4.5 mg/mL	12.5	
	EG-II	39.0 23.0	+	4.8	3.0-3.4	55-60	25.0 "	33.3	
	EG-III	62.5 61.0	+	4.1	5.0	55	3.2 "	22.2	
	EG-IV	54.0 54.0	+	3.7	5.0	50-55	4.8 "	23.8	
	EG-V	44.5 44.0	+	4.0	4.8-5.2	65-70	2.0 "	11.8	
Geotrichum candidum 3C	III	SE SDS 69.4 67.0	4.19	4.6	3.8-4.2	55	(with CMC) 8.7 g/L		Rodionova et al. (1990)
Humicola grisea var. thermoidea YH-78		75.0 (SDS)	9.4		5.0	50			Yoshioka et al. (1982)
Thermoascus aurantiacus		SDS GF					(CMC) [5]		Tong et al. (1980)
	I	[6] 78.0	5.5		4.5-5.0	65	3.9 mg/mL		
	III	34.5 33.0	1.8		4.5-5.0	75	1.9 mg/mL		
Irpex lacteus (Polyporus tulipiferae)		SDS SE							Kanda et al. (1976a,b)
	F-1	35.6			4.0-5.0	40	9.52 X 10^{-2} %	2.5 X 10^{-3} ΔA/min/mL	
	S-1 [7]	56.0 51.7	12.2		4.0-5.0	50			
Irpex lacteus (Polyporus tulipiferae)	E_n-1	15.5 (GF)	0.73		4.0	50	0.561 mM (G_5)	3.75 X 10^{-2}	Kanda et al. (1980)
							0.381 mM (G_6)	3.00 X 10^{-2} ΔA_{660}/min	
Irpex lacteus (Polyporus tulipiferae)	E_2-A [8]	60.0			4.0	60			Kubo and Nisizawa (1983)
	E_2-B	35.0			4.0	60			
Favolus arcularius	I								Enokibara et al. (1992)
	II								
	III_a	28.0 (SDS)		5.08	4.5	50	0.28% (CMC)		
Chaetomium thermophile var. dissitum	41.0		4.53			66 (CMC)			Eriksen and Goksøyr (1977)

Table 2. Physicochemical characteristics of isolated fungal and bacterial endoglucanases

Source	Enzyme	M.W. (kDA)	Carbohydrate content (%)	Isoelectric point	Optimum pH	Optimum temperature (°C)	Km	V_{max}	References
Sporotrichum pulverulentum [9]	T_1 T_{2a} T_{2b} T_{3a} T_{3b}	32.3 (SE) 36.7 (SE) 28.3 (SE) 37.5 (SE) 37.0 (SE)	10.5 0.0 7.8 4.7 2.2	5.32 4.72 4.40 4.65 4.20			4 g/L 2 g/L 3 g/L 9 g/L 4 g/L		Eriksson and Pettersson (1975) Almin et al. (1975)
Sporotrichum (Chrysosporium) thermophile ATCC 42464	endo I[10] endo II endo III		+ + -	5.1-5.2 4.15-4.25 5.65-5.75					Canevascini et al. (1983)
Robillarda sp. Y-20		SDS GF 59.0 56.5	---	3.5	4.0-5.0	55	1.2		Uziie and Sasaki (1987)
Neurorspora crassa cell-1 mutant	Endo I Endo II Endo III Endo IV	46.5 (SDS) 21.0 (SDS) 37.3 (SDS) 30.0 (SDS)	29.4 5.3 6.4 6.4	 6.5 5.5	4.0 4.0 4.5 6.0	55 55 50 45			Yazdi et al. (1990a,b)
Sclerotinia sclerotiorum	EG 1 EG 2	SDS GF 48.0 46.0 34.0 32.0		6.2 3.7	5.5 5.5	55 50	(CMC) (MUFC) 20.6[11] 2.1 8.7 3.6	(CMC) (MUFC) 0.09[12] 0.22 0.80 0.017	Waksman (1991)
Actinomycetales									
Microbispora bispora	Endo I Endo II	44.0 (SDS) 57.0 (SDS)		4.8 4.2					Yablonsky et al. (1988)
Thermomonospora fusca	E_1 E_2 E_3 E_4 E_5	100.0 (a.a.) 41.5 (a.a.) 65.0 (SDS) 106.0 (SDS) 46.3 (a.a.)	- + + - -	3.2 4.7 3.1 3.6 4.5					Wilson (1992)
Anaerobic Bacteria									
Bacteroides succinogenes S85 [13]	EG1 EG2	65.0 (SDS) 118.0 (SDS)		4.8 9.4	6.4 5.8	39 39	(CMC) 3.6 mg/mL 12.2 mg/mL	84 U/mg 10.5 U/mg	McGavin and Forsberg (1988)

Table 2. Physicochemical characteristics of isolated fungal and bacterial endoglucanases

Source	Enzyme	M.W. (kDA)	Carbohydrate content (%)	Isoelectric point	Optimum pH	Optimum temperature (°C)	Km	V_{max}	References
Acetivibrio cellulolyticus	C_2 C_3	33.0 (SDS) 10.4 (SDS)							Saddler and Khan (1981)
Ruminococcus albus F-40		50.0			6.7	44	(with CMC) 7.2 (DS=0.6) 0.7 (DS=0.95) 0.4 (DS=1.4)		Ohmiya et al. (1987)
Ruminococcus flavefaciens strain 67		> 3000.0 89.0			6.4 6.4	45 45			Pettipher and Latham (1979)
Clostridium josui		45.0 (SDS)			6.8	60	15.4 (w/ G_4) 6.7 (w/ G_5)	1.4 (w/ G_4) [14] 8.1 (w/ G_5)	Fujino et al. (1989)
Clostridium stercorarium	I	100.0 (GF)		4.6					Bronnenmeier and Staudenbauer (1988)
Clostridium stercorarium		SDS GF 91.0 99.0	2	3.85	6.4	60	7.14 mg/mL (CMC, DS=0.75)	92 IU/mg	Creuzet and Frixon (1983)
Clostridium thermocellum LQR1		83.0-94.0	11.2	6.72	5.2	62	2.3 mM (G_5) 0.56 mM (G_6)		Ng and Zeikus (1981)
Clostridium thermocellum NCIB 10682		56.0		6.2	6.0	80			Petre et al. (1981)
Aerobic and Facultatively Anaerobic Bacteria									
Bacillus subtilis AU-1		23.0			5.5		4.0 (CMC)		Au and Chang (1987)
Bacillus sp. KSM-522	E-I E-II E-III	SDS GF 78.0 49.0 61.0 34.0 61.0 35.0		4.4 3.5 3.5	6.0 7.0-10.0 7.0-10.0	60 50 50			Okoshi et al. (1990)

Table 2. Physicochemical characteristics of isolated fungal and bacterial endoglucanases

Source	Enzyme	M.W. (kDA)	Carbohydrate content (%)	Isoelectric point	Optimum pH	Optimum temperature (°C)	Km	V_{max}	References
Bacillus sp. KSM-330	Endo K	SDS GF 42.0 39.0		> 10.0	5.2	45			Ozaki and Ito (1991)
Bacillus sp. KSM-635	E-H E-L	130.0 100.0	2-3 2-3	<4.0 <4.0	9.5 9.5	40 40			Yoshimatsu et al. (1990)
Cellulomonas sp. UQM 2903	I II III IV V VI	62.9 (GF) 44.3 (GF) 62.9 (GF) 76.9 (GF) 142.9 (GF) 120.6 (GF)			- 7.0 - 7.0 7.0 7.0	- 40 - 50 50 40-50	- 6.67 (CMC) [15] - 5.55 (CMC) 24.39 (CMC) 3.13 (CMC)		Prasertsan and Doelle (1986)
Cellulomonas flavigena ATCC 482		40.0			7.0		7.5 (CMC, DS=0.65-0.85)		Thayer et al. (1984)
Sporocytophaga myxococcoides	I II	46.0 (SDS) 52.0 (SDS)		7.5 4.75	6.5-7.5 5.5-7.5				Osmundsvag and Goksøyr (1975)
Erwinia chrysanthemi strain 3665	Z	45.0		4.3	6.2-7.5	52			Boyer et al. (1984).

(1) K_m was expressed in mM and V_{max} was expressed in µMol. min^{-1}. mg^{-1}
(2) V_{max} was expressed in µg . min^{-1} . $µg^{-1}$
(3) K' values calculated by the method of Hill
(4) V_{max} was expressed in mg . min^{-1} . mg^{-1}
(5) multiple bands were observed
(6) CMC with D.S.=0.75 and D.P.=3200
(7) S-1 is referred to as an endocellulase with Avicelase activity
(8) E-2 is considered an endocellulase of "Avicelase" type
(9) Sporotrichum pulverulentum is the same organism previously called Chrysosporium lignorum
(10) Endo I and III were more efficient hydrolysing crystalline cellulose, while Endo II showed only CMCase activity
(11) K_m was expressed in mg/mL in the assay with CMC and in mM in the assay with MUFC
(12) V_{max} was expressed in µM . min^{-1} . mg^{-1}
(13) Bacteroides succinogenes is currently named Fibrobacter succinogenes
(14) V_{max} expressed in µM . min^{-1} . mg^{-1}
(15) K_m expressed in mg/mL

Abbreviations SDS = Polyacrylamide gel electrophoresis with sodium dodecyl sulfate; LS= Light Scattering; GF= Gel Filtration; SE= Sedimentation Equilibrium; a.a.= amino acid sequence of protein

TABLE 3

Summary of the properties of the cellulolytic complexes of anaerobic bacteria

Organism	Surface location	Polycellulosome Diam. (nm)	Polycellulosome Mass million Da	Cellulosome Diam. (nm)	Cellulosome Mass million Da	Number by SDS	Subunits Number by TEM	Size range (K)	Calcium required	DTT required
A. cellulolyticus	--	--	--	--	--	--	--	20-210	√	√
B. succinogenes	√	60	--	--	4.5	20 +	--	20-200	--	√
C. thermocellum JW20	√	60	50-80	16-18	2.0-2.5	20 +	35	45-200	√	√
YM4	--	--	--	23-30	3.5	--	45-50	45-200	√	√
YS	√	--	--	18	2.1	20 +	--	45-210	√	√
ATCC 27409	--	--	--	--	6.5	--	--	--	√	√
R. albus	√	50-60 200-300	--	--	> 1.5	--	--	-200	--	√
R. flavefaciens	√	--	--	--	> 3.0	--	--	--	√	√

3. *IDENTIFICATION OF ENDO-TYPE ACTIVITY OF CELLULASES*

As cellulases are likely to have overlapping substrate specificities, it is impossible to determine precisely the activity of a particular enzyme of a cellulase complex, without first purification. To partially overcome this problem it is common to measure the production of reducing sugars by the action of cellulases upon substrates with different susceptibility to hydrolysis. However, it is generally accepted that the viscometric assays are more specific for assessment of EG activity.

- Polymeric substrates

Endoglucanases are, generally, found active on amorphous cellulose and cellulose derivatives, but inactive against crystalline cellulose. Nevertheless, EG and CBH acting together cooperate to hydrolyze crystalline cellulose (Coughlan and Ljungdahl, 1988). Fujii and Shimizu (1986) reported that only in the early stages of soluble cellulose hydrolysis endo- and exocellulases act synergistically. The action of EGs could be neglected when the substrate has MW below 4000.

It is accepted that the use of soluble cellulose derivatives enables the division of cellulases in two groups: endo- and exo-acting enzymes. EGs depolymerize these soluble substrates in a random manner, while CBHs seem to stop their attack at the first substituent on the polymer chain, presumably from the non-reducing end. However, the inability of an enzyme to attack soluble cellulose derivatives should not mean that, with insoluble non-substituted cellulose, it must behave as an exo-type enzyme (Biely, 1990).

Viscometric assays based on the reduction of the degree of polymerization of soluble cellulose derivatives (such as CMC, HEC) is a commonly used assay to characterize endo-acting cellulases.

The use of CMC for assays of EG activity has an enormous popularity, due to its soluble nature. Some investigators suggest that enzymes that act on CMC should be referred to as "endoglucanase (CMCase)" rather than as "cellulase". Particularly because CMC, concerning its solubility and noncrystallinity, could be regarded as more similar to cellodextrins than to cellulose (Weimer, 1992). However, other authors recommend to avoid names such as CMCase, because this imply that CMC is a natural substrate for these enzymes, which is not (Enary, 1983).

The traditional use of graphical comparisons of viscosity reduction and end-reducing groups production during the EGs action on CMC provides a measure of the "degree of randomness" of EGs (Kanda et al., 1976). Connely and Coughlan (1991) also agreed that the rate of increase in

fluidity and of the release of reducing sugars may be taken as an evaluation of the degree of randomness of the enzyme under study.

- Low molecular weight substrates

As it was already pointed out, cellulase system components and their isoforms are differentiated on the basis of their mode of action with a range of cellulosic substrates and their substrate specificity. An attempt to overcome the problems of poorly definition of polymeric cellulose substrates was directed towards the use of cello-oligosaccharides. The differentiation of cellulases achieved considerable progress with the introduction of chromogenic and fluorogenic substrates (van Tilbeurgh and Claeyssens, 1985; Claeyssens,1988). Substrates such as methylumbelliferyl cellobioside (MUC) and p-nitrophenyl-β-D-cellobioside (pNPC) have been used to evaluate exo-acting cellulase activity.

- Differences in substrate specificity among endoglucanases

A reliable differentiation of the components of cellulolytic systems according to their substrate specificities is important not only to characterize a particular system under study, but may contribute significantly to a better understanding of the mechanisms of cellulose degradation (Biely, 1990).

Bhat et al. (1989) reported that among the five major EGs isolated from *Penicillium pinophilum/funiculosum* there was difference in the mode of action depending on the substrate used. The smallest cello-oligosaccharide attacked by EG I and EG V were cellohexaose and cellopentaose, respectively. EG I and EG V showed a remarkable specificity on these soluble small substrates. Cellotriose and higher oligosaccharides were hydrolyzed by EG III and EG IV, cellotetraose was hydrolyzed by EG II. Also, EG I and EG V decreased very rapidly the viscosity of CM solutions and reduced the D.P. of H_3PO_4-swollen cellulose, causing only small effect on the reducing power of the solution. On the contrary, EG III and EG IV effected small changes on the viscosity or D.P. of CMC and H_3PO_4-swollen cellulose, but caused a dramatic increase on the reducing power of the solution. It was suggested that EG I and EG V are more randomly acting enzymes, attacking preferentially on internal bonds remote from the ends, while EG III and EG IV act in a less random manner, attacking the cellulose chains preferentially from the ends. EG II was intermediate in its catalytic action.

Reduced cello-oligosaccharides (Gum and Brown, 1977) and carbanilated (Bjorkqvist, 1981) normal and reduced sugars have also been used in the determination of mode of action of EGs.

Bhat et al (1990) had evidences that modification on the reducing glycosyl residue on the cello-oligosaccharides substrates may induce in apparent changes in the EGs mode of action. Using carbanilated normal and reduced substrates, the authors observed remarkable diversity in modes of action of EGs from *Penicillium pinophilum*. For example, EG I does not attack cellopentaose but attacks cellopentaitol, while EG V does not hydrolyze cellopentaitol but degrades cellopentaose. When methylumbellilferyl pentaoside was used as substrate both enzymes (actually all major EGs from *Penicillium pinophilum*) showed activity. The authors suggested that although cello-oligosaccharides derivatives have been useful for the differentiation and classification of EGs, the results of these analyses should be treated cautiously, concerning eventual conclusions on the mechanism of cellulase action. The same authors reported a successful use of radiolabelled cello-oligosaccharides of D.P. 3-5 for obtention of unequivocal information on the mode of actin of EG from *Penicillium pinophilum*.

These are indications of the difficulty in the selection of substrate for assessment of EG activity. To avoid puzzles and eventual problems the most appropriate approach should include a few different types of substrates, such as cello-oligosaccharides and a polymeric substrate. Nevetheless, it is still argueable that soluble cellulose derivatives (CMC, HEC, etc) are not appropriate substrates, because of their solubility and lack of crystallinity. It seems that these points are more relevant when the focal point is to evaluate the "complete celllulase activity", instead of simply determine EG activity. However, despite the common acceptance that CMCase activity is seen as EG activity, the definitions are becoming blurred, since some purified CBHs from *Trichoderma reesei* show appreciable activity against CMC (Bhat et al., 1989). Beldman et al. (1985) reported an interesting anomaly on a *Trichoderma viride* EG, which was inactive on CMC, but hydrolyzed crystalline cellulose. It has been considered that CBH II from *Trichoderma reesei* also has an endoglucanase-type of action (Enary and Niku-Paavola, 1987). CBH II does not hydrolyze cotton to soluble substrates, but contributes to an increase of the substrate ability to swell in alkaline media. Enary and Niku-Paavola (1987) suggested that CBH I and CBH II act synergistically in the hydrolysis of native crystalline cellulose, and that EG I and EG II accomplish only the hydrolysis of soluble cellodextrins available. Waksman (1991) reported that EG I, one of the two major endoglucanases purified from the plant pathogen *Sclerotinia sclerotiorum* exhibited also exo-activity, which was determined towards 4-methylumbelliferyl-cellobioside.

Although the use of systematic names for the cellulases implies that the various enzymes fall neatly into one or another category, some more recent literature reveals that this is very far from being the case. Indeed, if some of the views (Chanzy et al., 1983; Eriksson and Wood, 1985; Henrissat et

al., 1985) propounded on the mode of action of the enzymes described as CBH and EG are proved to be tenable, a complete reclassification of the enzymes may be necessary (Wood, 1991).

Specificity mapping of cellulolytic enzymes was carried out by Claeyssens and Henrissat (1992) corroborating the classification of cellulases (Henrissat et al., 1989) based on hydrophobic cluster analysis of amino acid sequences. Claeyssens and Henrissat (1992) described the presence of CBHs in three cellulase families (B, C and F) together with typical EGs. For these enzyme families, the differentiation between endo- and exo-acting enzymes cannot be accomplished with the use of small soluble substrates.

Wood (1991) concluded that "unequivocal and reproducible evidence for the substrate specificity of the individual enzymes appears to be difficult to obtain".

Despite the usefulness of soluble substrates for classification and identification of EGs, it is not at all certain if there is a parallel between the action on insoluble and soluble substrates (Wood, 1992).

Coughlan (1990) stated that the distinction between enzymes with endo- and exo-type of activity is not as simple as it was considered heretofore, and that there is an urgent need for the development of a more informative nomenclature.

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